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THE DIURNAL CYCLE OF THE POWDERY MILDEW ERYSIPHE POLYGONI[1]

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INTRODUCTION

Studies of the strains of *Erysiphe polygoni* DC. parasitizing red clover (*Trifolium pratense* L.), cabbage (*Brassica oleracea capitata* L.), and larkspur (*Delphinium* sp.) have shown that certain phases of fungus activity regularly occur at a certain time during the day. This developmental rhythm is called the diurnal cycle of *E. polygoni*, and its manifestation with respect to the germinability of the conidia, the formation of appressoria, and the formation and dissemination of conidia is described herein. Certain other phenomena, such as the time of day at which infection may occur and the transpiration of diseased leaves, also show a diurnal cycle. The cause and some of the limitations of this cycle are discussed.

GERMINABILITY OF CONIDIA

Conidia from field clover plants were shaken onto dry glass slides at 2- or 3-hour intervals throughout the day. Of four slides of conidia collected at each time, two were left fully exposed to the natural environment in the field and the other two were placed in Petri dish moist chambers held at 23° C. in the dark. Of 200 or more conidia on each slide, those germinating were counted on one slide of each pair after 3 hours (germination interval) and on the other slide after 6 hours. The results of one such test on August 22 to 23, 1933, are given in columns 2, 3, 4, and 5 of table 1. In this, as in other tests, conidia taken from field plants during the light portion of the day gave a higher germination than those taken at night. Conidia collected during the night, late morning, and early afternoon and exposed in the field showed little or no germination after either 3 or 6 hours; those exposed to the heat of the day were severely shriveled, but those exposed at night, when the temperature was much lower, remained turgid. Conidia collected between 10 a. m. and 8 p. m. germinated well in the moist chambers at 23° C., while those collected at night germinated poorly or not at all. There was but little increase in germination after 3 hours.

In several tests the cycle of germinability of conidia from field plants was compared in light and in darkness. The spore mounts exposed to light were in Petri dish moist chambers 93 cm from eight

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300-watt lamps, in a constant environment of 22° C. The mounts in darkness were in light-proof paper boxes beside the exposed mounts. The temperature in the boxes was 1° to 2° higher than the outside temperature, but this slight difference probably had little or no effect on the results. The results of one of these tests are given in columns 6 and 7 of table 1. The conidia taken in the high phase of the germination cycle (noon to 7 p. m.) germinated almost as well in darkness as in light, but conidia taken in the low phase of the cycle were greatly stimulated by light.

TABLE 1.—*Effect of time of collection, of germination interval, and of environment on the germination of conidia of Erysiphe polygoni from field clover plants, Madison, Wis., 1933*

Time of making spore mount	Germination of conidia after indicated treatment on—					
	Aug. 22-23				Aug. 29-30	
	Spore mounts exposed in the field for—		Spore mounts held at 23° C. in dark moist chambers for—		In artificial light for 3 hours at 22° C.	In darkness for 3 hours at 23°-24° C.
	3 hours	6 hours	3 hours	6 hours		
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1 p. m.	[1] 0	[1] 0	50	57	79	40
3 p. m.	[1] 0	[1] 0	52	87	64	48
5 p. m.	51	83	56	64	38	25
7 p. m.	4	6	79	53		
8 p. m.	1	3	24	26		
9 p. m.					76	6
10 p. m.	0	0	6	6		
12 p. m.					32	0
1 a. m.	0	0	1	4		
3 a. m.					18	1
4 a. m.	0	0	0	1		
6 a. m.					29	3
7 a. m.	0	[1] 0	0	9		
9 a. m.					29	1
10 a. m.	[1] 0	[1] 0	15	70		
12 m.					89	32
1 p. m.	[1] 0	[1] 0	43	64		
3 p. m.					83	72

[1] Conidia severely shriveled.

The cycle of germinability of conidia from plants growing in the greenhouse and of conidia from plants growing in environmental control chambers was similar to that already described for conidia from plants growing in the field. In the greenhouse during these tests the temperature was nearly constant at 20° C., the relative humidity was not measured, and the duration of light was similar to that prevailing outdoors. In the control chambers the plants were grown at 20°, 60 percent relative humidity, and in a 12-hour day (12 hours' continuous light alternated with 12 hours' continuous darkness) with about 1,050 foot-candles of light at the level of the plants.

Age of conidia was not found to influence the cycle of germinability. In one test conidia from mildewed greenhouse plants from which most of the mature conidia had been removed by shaking the plants 24 hours before starting the germination test showed the same cycle of germinability as conidia from plants on which the conidia had been allowed to accumulate for 4 days.

Several attempts were made to increase the germination of conidia taken in the low phase of the germinability cycle. Exposure to light greatly increased the germination of these conidia, as already men-

tioned. Placing the conidia on sugar solution, placing the petioles of the mildewed leaves in 1-percent sucrose solution for 4 hours before making the germination test, and the presence of healthy host leaves in the germination chamber had no effect on the germination of conidia, but in each of five tests the germination of conidia was greatly increased by placing the conidia on host leaves. Excised leaflets from healthy greenhouse plants and dry slides were placed at random in the bottom of a large can, and conidia from mildewed greenhouse plants were dusted on them from above. The average number of conidia counted per unit area on the slide was assumed to be the same on the leaflets. The leaflets and slides were placed in Petri dish moist chambers during the germination interval. The germinated and the ungerminated conidia on the glass slides were counted after 9 hours. After the same period, which was sufficient to allow leaf penetration by the haustoria from the germinated conidia, the leaflets were fixed in a solution of alcohol and acetic acid, stained with acid fuchsin, and examined. The number of established young colonies per unit area of leaves was divided by the original number of conidia per unit area, as determined from the glass slides, to determine the percentage of germination on the leaves. The value obtained in this way was low because many germinated conidia were washed away in the staining process, but the results (table 2) show that in each case the amount of germination on host leaves was much greater than that on glass slides.

TABLE 2.—*Effect of host leaves on germination of conidia, Madison, Wis., 1933*

Germination interval	Germination of conidia on—	
	Dry glass slides in dark moist chambers	Excised clover leaflets in dark moist chambers
	Percent	*Percent*
12 p. m., Nov. 6, to 9 a. m., Nov. 7	4.2	19.20
12 p. m., Nov. 12, to 11 a. m., Nov. 13	.0	.11
11 p. m., Nov. 23, to 9 a. m., Nov. 24	3.2	21.90
10 a. m., Dec. 2, to 9 a. m., Dec. 3 [1]	2.9	15.50
10 a. m., Dec. 4, to 7 p. m., Dec. 5 [1]	1.0	25.00

[1] The low germination in the tests that were started at 10 a. m. is believed to be due to the cloudy winter weather which is unfavorable for mildew development and which was prevailing at this time.

The diurnal cycle of germinability of conidia appears to be a direct or indirect result of the alternation of light and darkness to which the mildewed plants are normally subjected in nature. When mildewed plants were grown in control chambers in a 12-hour day where light was the only environmental factor known to vary throughout the day, a definite diurnal cycle of conidial germination was apparent, as previously noted.

EFFECT OF LIGHT ON ELONGATION OF HYPHAE AND FORMATION OF APPRESSORIA

Plants grown in the greenhouse were inoculated and placed in the environmental control chambers at about 24° C. and 60 percent relative humidity for a 12-hour day. Samples of leaves were removed from the plants at about 6-hour intervals, and the lengths of hyphae

were measured and the appressoria counted in individual fungus colonies. The average size of the colonies and the number of appressoria were determined, after staining, from measurements and counts on each of 5 to 10 colonies on each of 4 to 10 leaflets removed at each time of sampling. The rate of growth of mildew colonies was followed in this way in three tests. These tests gave similar results. The data of one test are given in table 3. The length of hyphae increased at a fairly uniform rate in light and in darkness, but appressoria were formed almost entirely in the light only.

When inoculated plants were exposed to 6-hour periods of alternating light and darkness the rate of increase of colony size and number of appressoria was less than on plants in a 12-hour day, especially at the beginning of the test, and appressoria were formed in about equal numbers in light and in darkness.

TABLE 3.—*Elongation of the hyphae of Erysiphe polygoni and formation of appressoria in light and in darkness, Madison, Wis., 1934*

Time of sampling	Period between inoculation and sampling	Light condition of interval preceding measurement or count	Growth of fungus on leaves exposed to alternate 12-hour periods of light and darkness	
			Average length of mycelium in colony	Average number of appressoria per colony
	Hours		μ	
12 p. m., Jan. 20	16	Dark	83	[1] 1.0
7 a. m., Jan. 21	23	do	232	[1] 1.0
12 m., Jan. 21	28	Light	462	2.1
6 p. m., Jan. 21	34	do	683	4.2
12 p. m., Jan. 21	40	Dark	1,140	4.5
7 a. m., Jan. 22	47	do	1,780	5.0
12 m., Jan. 22	25	Light	2,465	9.2

[1] Primary appressorium from the germ tube.

In determining the effect of light on the rate of colony growth by the previous method, growth was measured as the total increase in the length of hyphae per colony on the sampled leaves, but the rate of elongation of individual hyphae tips could not be followed by that method. In another test young recently inoculated leaves were excised from plants and floated on water in dishes in the control chambers. Several individual hyphal tips were selected, their positions were marked by means of drawing at the beginning of the test, and the subsequent growth measured at intervals thereafter. In measurements of 30 hyphal tips at about 3-hour intervals throughout the 24 hours of the day, the hyphae grew at an average rate of about 20μ per hour, and no significant difference in the rate of growth in light and in darkness could be detected.

The total length of hyphae and the number of appressoria per colony were determined in two experiments in which the host plants placed in the control chambers were given light exposures of various lengths. The results of one experiment are given in table 4. At 32 hours after inoculation the fungus colonies on the plants held in continuous darkness after inoculation were smaller than those exposed to even 4 hours of light per day, and at 48 hours after inoculation the differences were very large. At 48 hours after inoculation the size of the colonies (the total length of hyphae) increased with the length of

light exposure from 699μ per colony in darkness to 1,880μ in a 12-hour day, and then decreased with increased light to 1,500μ in continuous light. The number of appressoria per colony showed the same general trend, increasing from 3.3 in continuous dark to 10.4 per colony in a 12-hour day, and falling to 2.1 in continuous light. The average length of hyphae per appressorium showed more clearly than did the size of the colonies or the number of appressoria per colony the effect of the length of day on mildew development. For example, on the plants in continuous darkness, the mildew colonies, which never reached the sporulation stage, developed an average of 212μ of hyphae per appressorium. On plants in 4, 8, 12, and 16 hours of light per day, the colonies, which later showed a well-marked diurnal cycle of sporulation, had from 165μ to 190μ of hyphae per appressorium. In contrast, the colonies developing on plants in light exposures of 20 and 24 hours per day, which showed a greatly disturbed cycle of sporulation, as described later, had 624μ and 714μ of hyphae per appressorium, respectively.

TABLE 4.—*Relation of length of day to mycelial elongation and appressorium formation of Erysiphe polygoni*

Light condition per day	Growth 32 hours after inoculation			Growth 48 hours after inoculation		
	Average total length of hyphae per colony	Average number of appressoria per colony	Length of hyphae per appressorium	Average total length of hyphae per colony	Average number of appressoria per colony	Length of hyphae per appressorium
	μ		μ	μ		μ
Continuous darkness	270	1.68	160	699	3.3	212
4 hours light	616	1.78	346	1,070	6.4	167
8 hours light	625	1.92	326	1,620	9.8	165
12 hours light	644	2.17	297	1,880	10.4	180
16 hours light	609	2.02	302	1,678	8.8	190
20 hours light	571	2.40	238	1,620	2.6	624
Continuous light	593	1.62	366	1,500	2.1	714

The increase in the size of the colonies as the period of exposure to light was increased from 0 to 12 hours per day is interpreted by the writer as mainly due to the nutritional conditions of the fungus, the increased exposure to light resulting in a greater supply of carbohydrates in the parasitized leaves. That light is in itself not necessary or even very stimulatory for mildew growth has been shown by dish-culture experiments in which some inoculated leaflets floating on sugar solutions were exposed to the natural light of the laboratory and others were exposed to total darkness. The mildew development was luxuriant and similar in both cases, although slightly better on the leaves exposed to the light. The decrease in colony size as the light exposure was increased from 12 to 24 hours per day may have been due in part to the few appressoria, and hence also the few haustoria through which the fungus could draw its food supply.

The small number of appressoria per colony in continuous darkness, while much less than for colonies in alternating light and darkness of 4 to 16 hours of light per day, is not greatly less when the size of the colonies is taken into consideration. The small number of appressoria per colony, however, both in absolute numbers and in proportion to the size of the colony in 20 and 24 hours of light per day appears at present to be a manifestation of a disturbed diurnal cycle of the fungus. It is interesting that while in a 12-hour day appressoria were formed almost entirely during the light period, yet in 20 or 24 hours of

light per day there were fewer appressoria formed per colony or per unit length of mycelium than in continuous darkness. Certainly, normal appressorium formation can hardly be a direct response to light, neither does it appear to be favored by darkness; it must be dependent on the alternation of light and darkness, which is necessary for the manifestation of the normal cycle of the mildew fungus.

FORMATION AND ABSTRICTION OF CONIDIA

Sporulation of clover powdery mildew begins about 5 days after a susceptible plant has been inoculated. The initiation of the conidiophores has not been followed by the writer, but the changes undergone by the mature conidiophores throughout the day have been observed on plants growing in the field, in the greenhouse, and in the control chamber. To observe the conidiophores, leaflets with young vigorous mildew colonies were folded of a slide with the undisturbed conidiophores on the upper surface on the leaf projecting beyond the

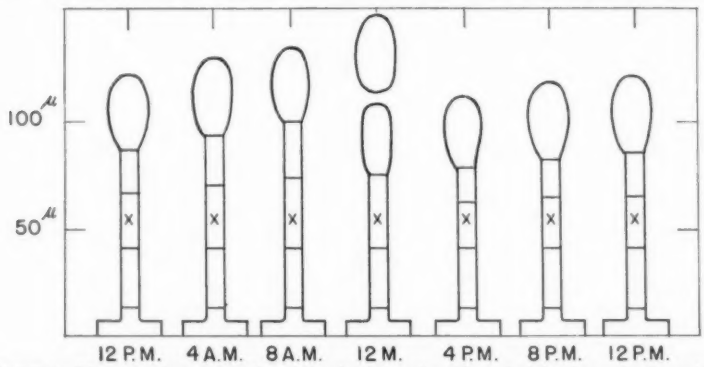

FIGURE 1.—Diagrammatic representation of the abstriction of conidia and of the changes in the conidiophore throughout the day, as observed on mildewed clover plants in the field at Madison, Wis., September 4, 1933. The generative cell (*x*) is the third cell from below.

edge of the fold, where they could be examined with the low- or high-power objectives of the microscope. The diurnal changes observed in the conidiophores on field plants on September 4, 1933 (13-hour day, sun rose at 5:25 a. m. and set at 6:28 p. m.), are illustrated in figure 1, and are quite typical of numerous observations made earlier and later in the season outdoors and in the greenhouse. The conidia were abstricted between 8 a. m. and 12 m. The abstricted conidium appears to remain passively attached to the conidiophore until it is dislodged by air currents or by disturbances of the leaves. After the abstriction of the conidium, the terminal cell of the conidiophore gradually increased in diameter (mostly during the light portion of the day) to form a new conidium which was abstricted the following morning. Thus each normally functioning conidiophore abstricts one conidium per day. The generative cell increased gradually in length and divided into two cells about 4 p. m. by a septum nearer its distal end. The basal cell showed no change in its gross morphology throughout the day.

Variations in this process are caused by seasonal and daily weather changes, but these have not been studied. However, on all days on

which the maturation of conidia was followed in the field in 1933 the conidia were not abstricted at 8 a. m. and were completely abstricted at 12 m. In the control chamber in a 12-hour day conidia were all abstricted 4 hours after the beginning of the light period.

The diurnal cycle of the abstriction of conidia is extremely definite and easily observed on vigorous mildew colonies and during clear weather. The developmental changes in the conidiophore are gradual, but the abstriction of conidia and the division of the generative cell are very specific processes which are generally complete on 90 percent of the conidiophores within the period of 2 hours for each process. In dull cloudy weather the diurnal cycle of the maturation of conidia is much less regular.

The diurnal cycle of the formation of conidia, like the diurnal cycle of conidial germinability, is dependent on the alternate light and darkness of the normal day. In the environmental control chambers with day lengths of 4, 8, 12, 16, and 20 hours, there was definite diurnal cycle of conidium formation, but in continuous darkness, in 2 hours of light per day, 22 hours of light per day, and continuous light no cycle of conidium formation was apparent. When plants subjected to a 12-hour day were given their daily light exposure from 8 p. m. to 8 a. m. instead of from 8 a. m. to 8 p. m., as was usually done, conidium formation followed a cycle, the various stages of which occurred 12 hours later than in the normal 12-hour day.

SPORE DISSEMINATION

Determinations of spore dissemination were made in the field during June, July, August, and September 1933, by counting the conidia caught during known intervals of time on slides placed in a field plot of mildewed clover. Three slides were placed on the ground at three different locations in the field and were changed at about 4-hour intervals during the day and less frequently at night. The determinations covered six series of observations of 1 day or more each, totaling 15 days in all. The results for 1 day or more in each of five of these series are given in table 5. In all cases the greatest liberation of spores occurred during the day, the maximum dissemination occurring about 12 m. This is a little later than the time of maximum spore maturation.

TABLE 5.—*Spore dissemination from mildewed clover plants in the field as determined by number of conidia caught at different times throughout the day and night*

Series	Period in which conidia were caught	Average number of conidia [1]
No. 1	2 a. m. to 6 a. m., June 16	0.05
	6 a. m. to 10 a. m., June 16	6.4
	10 a. m. to 2 p. m., June 16	15.6
	2 p. m. to 6 p. m., June 16	3.5
	6 p. m. to 10 p. m., June 16	.11
	10 p. m., June 16, to 8 a. m., June 17	.16
No. 2	2 a. m. to 4 a. m., June 28	5.0
	4 a. m. to 10 a. m., June 28	12.0
	10 a. m. to 12 m., June 28	39.1
	12 m. to 2 p. m., June 28	43.4
	2 p. m. to 4 p. m., June 28	61.2
	4 p. m. to 6 p. m., June 28	34.3
No. 3	8 p. m., July 5, to 8 a. m., July 6	11.2
	8 a. m. to 12 m., July 6	70.0
	12 m. to 4 p. m., July 6	58.7
	4 p. m. to 8 p. m., July 6	41.8
	8 p. m., July 6, to 8 a. m., July 7	21.9
No. 4	7 p. m., July 15, to 10 a. m., July 16	1.6
	10 a. m. to 1 p. m., July 16	47.4
	1 p. m. to 3 p. m., July 16	44.4
	3 p. m. to 6 p. m., July 16	25.4
	6 p. m. to 8 p. m., July 16	9.4
	8 p. m. to 10 p. m., July 16	3.1
	10 p. m., July 16, to 6 a. m., July 17	1.6
No. 5	9 p. m., Sept. 6, to 6 a. m., Sept. 7	.7
	6 p. m. to 12 m., Sept. 7	8.8
	12 m. to 4 p. m., Sept. 7	12.0
	4 p. m. to 7 p. m., Sept. 7	5.6
	7 p. m. to 11 p. m., Sept. 7	1.4
	11 p. m. to 7 a. m., Sept. 8	.7

[1] Caught per hour per square centimeter of slide.

EFFECT OF TIME OF INOCULATION UPON INFECTION

Field plants, potted plants, excised leaves with their petioles in water, and excised leaflets floating on sucrose solution have been used extensively in inoculation experiments. For the tests of the relative success of inoculations at different times during the day, most of the inoculations were performed by dusting healthy leaves with spores from field or greenhouse plants, but in some cases selected leaflets were inoculated by touching them with a heavily mildewed leaf. The severity of mildew resulting in 4 to 7 days was estimated by the method considered most satisfactory for the particular test. In some cases the individual leaflets were rated for their relative mildew severity and the values averaged for each unit of a test. With mass inoculations on potted plants the mildew was estimated for each pot as a unit. With inoculations on individual leaves on field plants, one leaflet was left uninoculated as a control on which to estimate contamination.

The results of these tests are given in table 6, where the values for mildew development in different tests are brought to the same basis by considering the mildew development on the optimum unit of each test as 10 and expressing the results of inoculations made at other periods in relation to this maximum infection.

TABLE 6.—*Effect on mildew development of the period of day when inoculations were made*

Date of test	Location	Host material	Relative amount of mildew resulting from inoculations at [1]—					
			1 to 5 a. m.	5 to 9 a. m.	9 a. m. to 1 p. m.	1 to 5 p. m.	5 to 9 p. m.	9 p. m. to 1 a. m.
1932								
June 24	Field	Excised leaves		0.5		10.0	6.6	0.7
July 2	do	do				7.5	10.0	4.5
Do	do	do		3.3	2.7	4.9	3.0	5.7
July 9	do	do				8.5	10.0	3.9
				2.9		7.2	4.7	2.9
1933								
June 5	do	Field plants		6.4		1.2	10.0	1.0
June 12	do	do				6.2	10.0	10.0
June 14	do	do				10.0	5.2	7.2
July 6	do	Potted plants				10.0	10.0	4.0
Aug. 25	do	Field plants	0.4			5.1	10.0	1.1
Do	do	Potted plants				10.0	5.2	5.8
Sept. 1	do	do	3.2	7.2	9.8	8.0	10.0	6.4
Sept. 18	Field and greenhouse [2]	do	1.5	8.5	10.0	7.5	1.0	1.5
Sept. 22	do [2]	do	1.8	4.1	10.0	5.3	1.8	2.3
Oct. 5	Greenhouse	do	1.4	7.5	5.6	10.0	2.6	1.4
Average			1.6	5.0	7.6	7.4	6.6	3.9

[1] 10 represents maximum infection.
[2] Average of 1 series in field and 2 in greenhouse.

The mildew development resulting in the different tests varied considerably. Some infection resulted from inoculations made at any time during the normal day, but inoculations made during the period of daylight were most successful. The success of inoculations made during the hottest and driest periods of sunny days illustrates the extreme tolerance of this fungus to low humidity.

In the previously reported tests of mildew infection throughout the light and dark portions of the day, the evaluation of the causes for differences in infection resulting from inoculations at different periods was difficult because of the simultaneous variation in the fungus, the host, and the environment. In a few experiments a more adequate separation of the influence of light from the other factors was obtained by inoculating two groups of plants simultaneously with the same inoculum and incubating them in a similar environment, except that one group was placed in the natural light and the other was placed in darkness for the first few hours or from the time of inoculation until nightfall, and afterward all were exposed to the same environment. The results of several such tests are given in table 7. In most cases heavier infection resulted on plants incubated in the light, but generally the difference was small and in some cases no difference was apparent.

TABLE 7.—*Effect of light on mildew infection in 2 groups of plants inoculated simultaneously*[1]

Time of inoculation	Host material	Additional environmental condition of host material	Relative mildew infection resulting in group kept in—	
			Light	Darkness
1932				
2 p. m., Nov. 19	Potted plants		10.0	3.0
Do	do	Humid bell jars	3.0	1.0
1933				
2 p. m., Feb. 2	Excised leaves	Petri dishes	4.2	2.5
2 p. m., Feb. 6	do	do	3.1	2.0
11 a. m., Feb. 21	do	do	3.1	1.8
12 m., Mar. 2	do	do	4.2	2.5
Mar. 30 [2]	Potted plants		9.5	7.5
Apr. 15 [2]	do		9.1	9.1
5 p. m., Apr. 17	do		9.0	9.0
5 p. m., Apr. 20	do		9.0	9.0

[1] Tests were made in the greenhouse.
[2] Average of 2 inoculations, 1 at 1 p. m. and the other at 5 p. m.
[3] 3 inoculations, 1 at 10 a. m., 1 at 2 p. m., and 1 at 5 p. m., did not give apparent differences due to light

The writer believes that infection (the establishment of a nutritional relation between host and parasite) of red clover with *Erysiphe polygoni* normally occurs in greatest abundance during the light portion of the day, and this belief is supported by the following facts: (1) Conidia were disseminated in greatest abundance during the light portion of the day; (2) germination of conidia was favored by light; (3) formation of appressoria was favored by light; (4) conidia taken from diseased plants during the light portion of the day germinated more readily than those taken at night; (5) the closure of clover leaves at night by the coming together of the two lateral leaflets and the movement of the center leaflet to a vertical position would reduce the chances of air-borne conidia falling on the leaves; (6) inoculations made during the day were more successful than those made at night; and (7) inoculations made in the light were more successful than inoculations made in darkness.

The relative importance of the various factors favoring infection during the light portion of the day cannot be determined from the present results, but all probably play a part. The dissemination of conidia during the light portion of the day and their rapid germination would be important. The fact that conidial germination is

greatly stimulated by host leaves would reduce the importance of the effect of light on germination and appressorium formation and of the diurnal cycle of conidial germinability as factors determining when infection might take place. The determinations of the dissemination of conidia and of conidial germination on dry slides in the field indicate that infection occurs principally in the latter part of the normal light period.

TRANSPIRATION OF MILDEWED AND OF HEALTHY CLOVER LEAVES

Leaves were excised from mildewed and from healthy greenhouse plants of the same age and general history except for inoculation, and were placed with their petioles in flasks of water (four leaves per flask), which were then tightly stoppered with cotton. The transpiration of five flasks of mildewed leaves and of five flasks of healthy leaves was followed under greenhouse conditions, and also that of five flasks of mildewed leaves and five flasks of healthy leaves in the constant light, temperature, and humidity of the control chamber. Weighings were made to one one-hundredth of a gram at intervals of about 3 hours and measurements of water loss were continued for 29 hours. As the ratio of dry weight to leaf area was not significantly different for healthy and mildewed leaves (0.434 ± 0.0095 g per square decimeter for healthy and 0.430 ± 0.0078 g per square decimeter for mildewed leaves) the results are expressed on a dry-weight basis. The average results of this test (table 8) show that in continuous light the transpiration of mildewed leaves was about the same as the transpiration of healthy leaves, and neither showed any diurnal variation. In the greenhouse, however, the transpiration of both diseased and healthy leaves showed the expected general variation between day and night, but while the transpiration of diseased leaves was similar to that of healthy leaves during the day, the transpiration of mildewed leaves was much greater at night. Four other tests in less detail gave similar results. In one test in which transpiration at 15° C. was compared with that at 30° the increased transpiration at night due to mildew infection was much greater at 15° than at 30°.

TABLE 8.—*Effect of mildew infection on the transpiration of clover leaves throughout the day, Dec. 11–12, 1933, at Madison, Wis.*

Time interval	Effect of infection on transpiration of clover leaves					
	In the greenhouse			In continuous light		
	Water lost per gram dry weight of leaves per hour		Increase or decrease in transpiration associated with mildew infection	Water lost per gram dry weight of leaves per hour		Increase or decrease in transpiration associated with mildew infection
	Healthy leaves	Mildewed leaves		Healthy leaves	Mildewed leaves	
	Grams	*Grams*	*Percent*	*Grams*	*Grams*	*Percent*
11:15 a. m. to 12:30 p. m.	5.78	5.60	−3.11	2.01	1.71	−14.9
12:30 p. m. to 3 p. m.	6.19	6.23	+.65	2.55	2.61	+2.35
3:30 p. m. to 4:50 p. m.	3.38	4.11	+21.6	2.36	2.16	−8.47
4:50 p. m. to 7 p. m.	1.06	1.71	+61.3	2.48	2.18	−12.1
7 p. m. to 9 p. m.	.67	1.13	+68.66	2.58	2.71	+5.04
9 p. m. to 11:30 p. m.	.64	1.17	+82.8			
11:30 p. m. to 7:30 a. m.	.84	1.51	+79.76	2.19	2.14	−2.28
7:30 a. m. to 10:25 a. m.	3.17	3.07	−3.15	2.14	2.25	+5.14
10:25 a. m. to 12:40 p. m.	4.81	4.76	−1.04			
12:40 p. m. to 3:05 p. m.	5.91	5.89	−1.33	1.72	1.81	+5.23

The resemblance between the effect of mildew on transpiration, as observed by the writer, and the effect of bordeaux mixture on transpiration, as observed by Duggar and Bonns [2] and others, is interesting. In both cases the effect is generally to greatly increase the transpiration of leaves during the night and to slightly decrease it during the light portion of the day. Because other phases of the development of clover mildew have been clearly shown to undergo a diurnal cycle as already described, the effect of mildew on transpiration may also possibly be due to a diurnal cycle of the fungus, but the work of Graf-Marin [3] would indicate that the cause of the day-and-night variation in the effect of mildew on transpiration was due to the smaller opening of the stomata of diseased leaves during the light portion of the day.

STUDIES OF OTHER POWDERY MILDEWS

Erysiphe polygoni was abundant on delphinium and on cabbage in the greenhouse in the fall and winter of 1933. These two forms of *E. polygoni* can be readily distinguished from the form on red clover and from each other by the size of the conidiophores and conidia, and by cross-inoculation tests. To the extent that they have been studied—namely, in the germinative capacity of the conidia and the development of the conidiophores—they show the same diurnal cycle as *E. polygoni* on red clover. Since this cycle was apparent on diseased plants which were exposed to constant temperature and humidity but to a 12-hour day, it appears that, as with *E. polygoni* on the red clover, the cause of the diurnal cycle of the fungus on cabbage and on delphinium is the alternation of light and darkness.

Erysiphe graminis DC., which is sharply different morphologically from *E. polygoni*, was also studied with respect to its development throughout the day and night. The basal cell of the conidiophore of *E. graminis* is decidedly swollen, and the transition from immature to mature conidia is gradual through several cells of the chain of conidia. For this reason it is difficult to trace the development of the conidiophore of this parasite in its relation to day and night. The results of several tests to determine whether *E. graminis* on barley showed a diurnal cycle in the germinative capacity of its conidia and in its ability to cause infection have shown no evidence of a definite variation between day and night. The germination of conidia was stimulated by light, however, as was the germination of the conidia of the other Erysiphaceae studied.

DISCUSSION

The writer's present conception of the diurnal cycle of *Erysiphe polygoni* is that certain phases of fungus development tend to occur during the light portion of the day and in some cases at a specific time of the day. Practically all the conidia formed during the 24 hours of the day are matured during the latter part of the morning (8 a. m. to 12 m.). The division of the generative cell occurs during a very limited period in the late afternoon and early evening. The

[2] DUGGAR, B. M., and BONNS, W. W. THE EFFECT OF BORDEAUX MIXTURE ON THE RATE OF TRANSPIRATION. Ann. Mo. Bot. Gard. 5: 153–176, illus. 1918.

[3] GRAF-MARIN, A. STUDIES ON POWDERY MILDEW OF CEREALS. N. Y. (Cornell) Agr. Expt. Sta. Mem. 157, 48 pp., illus. 1934.

dissemination of conidia, on the other hand, being presumably a passive process but dependent on the maturation of the conidia, on wind, and on the dryness of the leaf and fungus surface, is more or less continuous throughout the light portion of the day, but is less at night, perhaps because the humidity is higher then and there is less wind. Appressoria are normally formed during the light portion of the day, and they may be formed throughout the light period, but they form very readily in darkness under certain experimental conditions.

The greater activity of *Erysiphe polygoni* during the light portion of the day, and its ability to sporulate and cause infection under conditions of relatively low atmospheric humidity are in sharp contrast to certain downy mildews described by Weston,[4] in which the greatest activity of the fungus is in the dark and humid portion of the day. The basic cause of the diurnal cycle of an obligate parasite is difficult to determine, but the regular alternation of light and darkness appears necessary for the expression of the diurnal cycle of *E. polygoni*. The length of either the light or the dark portion of the day is of less importance, as indicated by the expression of the cycle in photoperiods of 4 to 20 hours. The great stimulatory effect of light on conidial germination on glass slides would indicate that the cycle might be due in large part to the direct effect of light on the fungus, but the luxuriant development of mildew on leaves in total darkness but floating on sucrose solution would not support this idea.

SUMMARY

Conidia of clover mildew (*Erysiphe polygoni*) germinated best when removed from diseased plants in the afternoon. With the onset of darkness the germinability of the conidia gradually decreased, reaching a minimum in the early morning but again rising with the onset of daylight. Attempts to increase the germination of conidia in the low phase of the germinability cycle have been successful only by exposing them to light or by placing them on host leaves.

The elongation of the hyphae of clover mildew continued at a fairly uniform rate throughout the day and night, but appressoria were formed principally during the light.

The generative cell of the conidiophore divided once each day in the late afternoon to form a new proximal generative cell and a distal daughter cell. One conidium was matured during the late morning on each conidiophore.

Conidia were caught on spore-trap slides in greatest abundance about noon, but there was a continuous dissemination of conidia throughout the middle and latter part of the daylight period, and few were caught at night.

The diurnal cycle manifest in the maturation of conidia and in the division of the generative cell was clearly evident on mildewed plants growing in 4, 8, 12, 16, and 20 hours of light per 24 hours, but in longer or shorter light exposures per day the conidiophores did not develop normally and the diurnal cycle was less evident or was no longer apparent.

Inoculations with clover mildew during the light portion of the day were more successful than inoculations made at night.

[4] WESTON, W. H. PRODUCTION AND DISPERSAL OF CONIDIA IN THE PHILIPPINE SCLEROSPORAS OF MAIZE. Jour. Agr. Research 23: 239–278, illus. 1923.

The transpiration of mildewed leaves was greater than that of healthy leaves at night and was about equal to or slightly less than that of healthy leaves during the day.

The diurnal cycles manifest in conidial formation, conidial germination, and appressorium formation were evident when temperature and humidity were constant, with plants growing in a 12-hour day, but these cycles, as well as that of transpiration, were eliminated when the test plants were grown in continuous light. It is concluded that the alternating light and dark of the normal day are responsible for the cyclic manifestations of *Erysiphe polygoni* described here.

So far as they were studied, the forms of *Erysiphe polygoni* on red clover, delphinium, and cabbage showed similar diurnal cycles, but no diurnal cycle of development was established with *E. graminis* on barley.

HOST RANGE AND PHYSIOLOGIC SPECIALIZATION OF RED CLOVER POWDERY MILDEW, ERYSIPHE POLYGONI[1]

By CECIL E. YARWOOD[2]

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INTRODUCTION

Since 1902, Neger (*7*)[4] and numerous other workers have shown that the morphologic species *Erysiphe polygoni* DC., parasitizing, according to Salmon (*8*), some 357 species in 157 genera, consists of a large number of physiologic races. Single races, may, though rarely, infect host species of different genera within a single plant family or may infect several species within a genus or, more rarely, may be limited to a single host species. Blumer (*1*), however, has divided the *Erysiphe polygoni* of Salmon into several species on the basis of the perfect stage. Of these, *E. martii* Lev., confined to species of the Leguminosae including red clover (*Trifolium pratense* L.), consists of several physiologic races (*2*).

The purpose of the present investigations was to add to the knowledge of the identity of the powdery mildew of red clover in the United States by studies of the host range and possible physiologic forms of the causative organisms.

HOST RANGE

The host range of the powdery mildew parasitizing red clover has been extensively investigated, but with extremely variable results. All investigators except Blumer (*1*), however, agree that the form found on red clover is limited to the host genus *Trifolium*. Salmon (*9*), Mains (*5*), and Blumer (*1*) used entire plants as hosts. Hammerlund (*2*) and Searle (*10*) used both entire plants and excised leaves in Petri-dish moist chambers. In a previous study the writer used entire plants in the field and the greenhouse, and excised leaves on sugar solution in dish cultures (*11*).

In the greenhouse and in dish-culture tests leaves of five plants of each host species were inoculated in most cases, and infection was considered positive if one or more plants of a species were infected. In the field a record was kept of the natural mildew infection of plants in a plot containing a number of *Trifolium* species adjacent to a plot of heavily mildewed red clover. That the mildew occurring on these *Trifolium* species in the field resulted from infection with conidia from red clover is not certain but appears very likely.

[1] Received for publication Oct. 7, 1935; issued June, 1936. Joint contribution from the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the botany department, Purdue University Agricultural Experiment Station.

[2] The writer wishes to express his indebtedness to E. B. Mains, formerly agent, Division of Cereal Crops and Diseases, and to A. J. Pieters, principal agronomist, Division of Forage Crops and Diseases, Bureau of Plant Industry, for assistance during the course of this work.

[3] Now associate in plant pathology, California Agricultural Experiment Station.

[4] Reference is made by number (italic) to Literature Cited, p. 664.

The greenhouse inoculations were performed by dusting young plants of the *Trifolium* species with conidia from mildewed red clover. No precautions against contamination were taken, but, as red clover powdery mildew was the only form of *Erysiphe polygoni* in the greenhouse at the time of these tests, contamination was unlikely.

In the dish-culture tests, leaflets were excised from mildew-free greenhouse plants, inoculated by touching their upper surfaces with a heavily sporulating mildewed leaf of red clover, and floated on 10-percent sucrose solution in Syracuse watch glasses in the diffuse light of the laboratory at about 20° C. Inoculated leaves were examined for infection about 10 days after inoculation.

In table 1 the *Trifolium* species tested are listed in the order of their apparent susceptibility, and the summarized results of other investigators are tabulated for comparison. It is difficult to express quantitatively the relative susceptibility of these species, but it may be said that *T. lupinaster* L. was almost if not quite as susceptible as *T. pratense*, that *T. hybridum* L. was much less susceptible, and that such species as *T. repens* L. and *T. incarnatum* L. were definitely resistant although sporulation of *Erysiphe polygoni* on excised leaflets of these species was observed in dish cultures. Each species tested in dish culture was inoculated separately with physiologic forms 1 and 2 of clover mildew, described later, but, since none of these species other than *T. pratense* showed any difference in susceptibility to the two forms of mildew, the results are not separately listed.

Leaflets of *Trifolium pratense* were inoculated in dish culture with conidia produced on each of the species listed as susceptible. Reciprocal inoculation was successful from all species but *T. incarnatum*.

The results given in table 1 emphasize the great variation in the results of different workers. In contrast to 1 susceptible species out of 8 tested by Salmon (*9*), 1 susceptible species out of the 3 tested by Searle (*10*), 1 susceptible species out of the 7 tested by Mains (*5*), 2 susceptible species out of the 8 tested by Hammerlund (*2*), and 4 susceptible species out of the 20 tested by Blumer (*1*), the writer found 14 species susceptible in varying degrees out of a total of 20 species tested. The reason for this extreme variation in the results of different investigators is unknown, but the following possible reasons are suggested:

TABLE 1.—*Infection of Trifolium species with conidia of Erysiphe polygoni from T. pratense* [1]

Host	Reaction of *Trifolium* spp. to *Erysiphe polygoni* according to—								
						Yarwood, 1931, La Fayette, Ind.			
								Dish cultures [3]	
	Salmon	Searle	Mains	Hammerlund	Blumer [2]	Field tests	Greenhouse tests	Plants inoculated	Plants infected
								Number	*Number*
T. pratense L.	+	+	+	+	+	+	+	5	5
T. lupinaster L.						+	+	4	4
T. carolinianum Michx.						+	+	2	2
T. striatum L.						+	+	3	3
T. reflexum L.			−			+	+	3	2
T. angustifolium L.						+	+	5	5
T. panormitanum Presl.						+	+	5	4
T. hybridum L.	−	−	−	−	+	+	+	4	3
T. squarrosum L.						+	+	5	5
T. resupinatum L.						−	+	5	4 2
T. subterraneum L.			−		−	−	+	5	4 4
T. fragiferum L.			−		+	−	+	5	4 5
T. repens L.	−	−	−	−	−	+	−	5	4 1
T. incarnatum L.	−		−			−	−	5	5 0
T. alexandrinum L.						−	−	5	0
T. dubium Sibth.	−			−	−	−	−	5	0
T. medium L.	−			+	+	−	−	5	0
T. pannonicum Jacq.					−	−	−	5	0
T. rubens L.					−	−	−	5	0
T. procumbens L.					−	−	−	5	0
T. agrarium L.	−				−				
T. badium Schreb.				−					
T. montanum L.	−				−				
T. thalii Vill.					−				
T. alpinum L.					−				
T. alpestre L.					−				
T. ochroleucum Huds.					−				
T. ambiguum Bieb.					−				
T. arvense L.				−	−				
T. trichocephalum Bieb.					−				
T. pallescens Schreb.					−				

[1] Plus sign (+) indicates that infection was microscopically visible.
[2] This column combines the results of 2 of Blumer's tests (1, *table 14*). If the results of these 2 tests differed, the positive infection was recorded here.
[3] Results of a test of Feb. 6, 1931. This test was repeated at 2 other times with essentially similar results.
[4] Weak infection.
[5] Weak infection was secured in a previous test.

(1) The use of different physiologic forms of the organism, variations in virulence, and differences in host range; (2) different environmental conditions, especially temperature; (3) vigor of the inoculum, including the germinability of the spores; (4) variation in the host species or inadequate representation of the species; (5) probable geographic variations in strains of *Trifolium*, which make positive results of much greater importance than negative results.

PHYSIOLOGIC FORMS

Salmon (*9*), Searle (*10*), Blumer (*1*), Hammerlund (*2*), and others have shown that the morphologic species *Erysiphe polygoni* of Salmon (*10*) consists of a number of physiologic races each of which is generally limited to a single genus or species of host plant. While these physiologic races may be indistinguishable morphologically in their perithecial stages, it is likely that most so-called physiologic races or forms of *E. polygoni* can be distinguished from each other by

the morphology of their conidial stages. Homma (*3*) has shown that there are appreciable morphological differences between the biological forms of *E. graminis* DC. on wheat, hull-less barley, *Poa annua* L., and *Elymus mollis* Trin. The writer has observed that the forms of *E. polygoni* (according to Salmon) on bean, red clover, California poppy, mustard, pea, cowpea, buckwheat, and columbine can be distinguished readily from each other by the gross morphology of their conidiophores and conidia. In the sense that they are clearly distinct morphologically in their conidial stages, these forms occurring on different host genera are probably not true physiologic forms. Moreover, Blumer (*1*) has distinguished many of these in Europe by the morphology of their perfect stages.

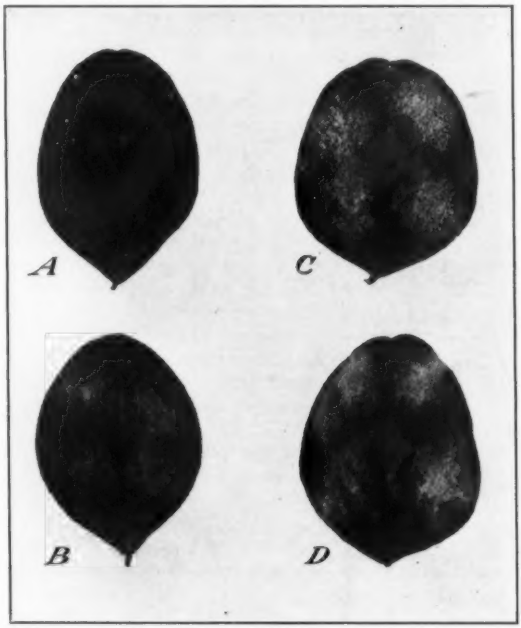

FIGURE 1.—Differentiation of physiologic forms 1 and 2 of clover mildew: *A*, Plant 23 inoculated with physiologic form 1; *B*, plant 23 inoculated with physiologic form 2; *C*, plant 2 inoculated with physiologic form 1; *D*, plant 2 inoculated with physiologic form 2. Plant 23 is immune from form 1 and susceptible to form 2; plant 2 is equally susceptible to both forms. Photographed 11 days after inoculation.

In July 1930 two clones of red clover (22 and 23), previously selected for mildew resistance, became heavily infected with powdery mildew. Healthy leaves of these and other red clover clones were taken from plants which had been protected from infection by dusting with sulphur, the leaflets were washed and inoculated with five collections of red clover powdery mildew in dish culture. Four of the six clones used were equally susceptible to the five mildew collections tested at this time, but clones 22 and 23 were immune (reaction with necrotic spots) (*6*) to the mildew prevalent in the field and in the greenhouse but were very susceptible to the inoculum taken from the mildew occurring on them. On the basis of this and a later test, two physi-

ologic forms of red clover mildew were distinguished. The mildew which was prevalent in 1930 and 1931 and to which clones 22 and 23 were immune is designated physiologic form 1, and the mildew to which these clones are susceptible is called physiologic form 2. Figure 1 illustrates the mildew reactions by which physiologic forms 1 and 2 of clover mildew were distinguished. Single-spore cultures of forms 1 and 2 of clover mildew were isolated and cross-inoculations repeated with similar results.

In the fall of 1931, a third physiologic form of clover mildew was found on a plant which was very resistant (reaction 1 with necrotic spots) (*6*) to forms 1 and 2. Except for clearly differentiating it from forms 1 and 2, form 3 has not been studied.

All determinations of physiologic forms of clover mildew were made in dish cultures. In the determination of the physiologic forms of many parasitic fungi, for example the wheat rusts genetically pure lines of the host plants are generally available, and a number of plants of each variety can be inoculated with each of the collections of the parasite to be tested. With such a heterozygous plant as red clover, however, this is not possible, unless the desired differential plants are multiplied by cuttings. This is a slow process, as only a limited number of cuttings can be secured from a given plant at any one time. Also, with a wind-disseminated fungus such as clover mildew, which produces conidia germinating readily in the absence of free moisture, careful greenhouse methods would be necessary to insure against contamination if physiologic forms were to be differentiated on potted plants. These difficulties were readily overcome by the use of dish cultures. If leaflets from the same plant are distributed in different dishes, a single plant may furnish a large number of closely comparable units for a given test, and the same plant may be used repeatedly as a source of mildew-free leaflets. Each unit of a test is enclosed in a glass chamber during the incubation period and thus protected from mildew contamination.

About 800 individual clover plants have been tested for their susceptibility to forms 1 and 2 of clover mildew. No plants susceptible to form 1 and resistant to form 2 have been found, and most of the plants which were more susceptible to form 2 than to form 1 were from crosses involving one or both of the clones on which form 2 was found.

During 1930 and 1931 collections of clover mildew were secured from British Columbia, Canada, and from Maine, New York, Michigan, North Carolina, Kansas, North Dakota, Idaho, and Washington, and seven collections were made in Indiana. These were tested on a series of host plants, including differentials for physiologic forms 1 and 2 of clover mildew, and in most cases for form 3. All collections, except one from Idaho and one from Indiana, corresponded to physiologic form 1 and did not contain forms 2 or 3. In the two exceptions in which form 2 was present, form 1 also may have occurred, since the differentials and methods in use would not determine clearly the presence of form 1 in a mixture of forms 1 and 2. It appears from these limited tests that form 1 is widely prevalent and that forms 2 and 3 are relatively rare. Moreover, since these three forms are similar, they are probably all of the epidemic type which spread so rapidly from the East to the West of the United States from 1921

to 1924.[5] Since powdery mildew of clover was present in the United States previous to 1921,[6] this earlier mildew was probably different from those studied.

Hammerlund (*2*) secured negative results when red clover was inoculated with the powdery mildew occurring on alsike clover (*Trifolium hybridum*), and Salmon (*8*), Mains (*5*), and Hammerlund (*2*) found alsike clover to be resistant to red clover mildew. However, Blumer (*1*) secured infection of alsike with the mildew occurring on red clover, and in Idaho (*4*) reciprocally successful inoculations of the mildews on red and alsike clover have been performed. In the present investigation alsike clover was successfully infected with physiologic forms 1 and 2 from red clover. Powdery mildew on alsike clover at La Fayette, Ind., also was tested on red clover plants and found to correspond to form 1 of red clover mildew.

No differences in the conidiophores and conidia of the three physiologic forms of red clover mildew could be detected, but it is possible that with more refined methods significant variations may be found between the forms. The presence of different forms can be readily determined at any time with certain host plants, but since form 2 was identified by its ability to infect certain plants immune to form 1 the same identical physiologic strains could not be described certainly as the result of future inoculations, except upon the use of identical host plants from which the forms were first described.

SUMMARY

Fourteen *Trifolium* species out of 20 tested in the field, in the greenhouse, and in dish cultures showed infection in varying degrees to red clover powdery mildew, *Erysiphe polygoni* DC.

Three physiologic forms of red clover powdery mildew have been distinguished by differences in the reaction of certain red clover clones.

These investigations were greatly facilitated through the use of a method of growing the clover mildew fungus on living excised leaves floating on sugar solution.

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CHRONIC PROGRESSIVE PNEUMONIA OF SHEEP, WITH PARTICULAR REFERENCE TO ITS ETIOLOGY AND TRANSMISSION [1]

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INTRODUCTION

The existence of a fatal lung disease of sheep has been recognized among the herds of the northwestern section of the United States, especially in Montana and Oregon, for at least two decades. Since the most prominent symptom of the disease is difficult, or labored, breathing, sheep so affected have been commonly referred to by sheep owners and herdsmen as "lungers."

Marsh (*5*)[2] made a study of the disease in Montana and published the first report of his findings in 1922. He found the disease to be of the nature of a chronic progressive pneumonia, the lesions in all cases being confined to the thoracic cavity. A disease very similar in character, known as "jagziekte", which has been recognized among the sheep of South Africa for many years, has been reported by Robertson (*8*), Mitchell (*7*), Cowdry (*1*, *2*), and others. Because of the striking similarity of the lesions of jagziekte of South Africa and chronic progressive pneumonia of Montana, Cowdry and Marsh (*3*) made joint comparative studies of the two diseases. They reached the conclusion that they were probably identical.

Chronic progressive pneumonia is unquestionably of considerable economic importance. Marsh (*5*) estimated that the losses from the disease among Montana sheep range from 2 to 10 percent, varying somewhat in the different herds. The mortality is generally believed to be 100 percent of the animals affected. Observations have indicated that the losses are greater among older animals, but sheep of all ages appear to be susceptible.

The principal objectives of the investigation reported in this paper were to determine the etiological factor or factors involved, the possibility of transmission, and other facts concerning the disease.

SYMPTOMS AND REPORTED CAUSES OF THE DISEASE

Because of the insidious nature of chronic progressive pneumonia of sheep, it is not known how long the animals are affected before the first clinical symptoms can be observed. The first noticeable symptom is labored breathing after exercise. As the disease progresses there is an acceleration of respiration, dilated nostrils, and flank breathing or pumping, even while the animal is at rest. More or less coughing, with some nasal discharge, has been observed in some cases. In the later stages the affected sheep show marked weakness and emaciation. Breathing becomes more difficult and the disease eventually terminates in death.

[1] Received for publication Jan. 6, 1936; issued June, 1936.
[2] Reference is made by number (italic) to Literature Cited, p. 679.

Several theories as to the cause of this type of chronic pneumonia in sheep and the similar disease jagziekte have been presented by different investigators.

Marsh (*6*), in his paper on bacteriological studies of chronic progressive pneumonia, reports the frequent isolation from the affected lung tissues of a diphtheroid which he considered an etiological factor, being either associated with, or secondary to, certain other possible predisposing factors. Robertson (*8*), who was one of the earlier investigators of jagziekte in South African sheep, believed the causative factor in that disease to be of the nature of a protozoan. Mitchell (*7*), in his studies, pointed to a specific virus as the cause of jagziekte. Cowdry and Marsh (*3*), in their later studies of the two diseases, reached the conclusion that although these chronic pneumonias, of which the epithelian lesions are particularly characteristic, may be due primarily to a specific cause, the pathological complex as a whole is such as might be caused by a variety of predisposing and exciting factors operating over a period of time.

SHEEP USED IN EXPERIMENTS

W. J. Butler, State veterinarian of Montana, at whose request these investigations were first undertaken, cooperated with this Bureau in furnishing, from that State, sheep affected with chronic progressive pneumonia. The sheep were shipped from Montana to the Experiment Station[3] of the Bureau at Bethesda, Md., where most of the transmission experiments were carried on. At various intervals during the course of the investigations the sheep were received in lots of 2, 2, 4, 6, 6, and 10, making a total of 30 sheep received from Montana Of this number 2 died in transit, 2 were found dead in the pens, and 3 showed only slight pneumonic lesions, leaving 23 to be used in the experiments.

For use in exposure tests, 96 normal sheep were obtained. These were native animals from Maryland. They were in fair condition, and most of them ranged in age from 1 to 1½ years.

OUTLINE OF METHOD

Frequent clinical observations were made on the diseased sheep from Montana, and as the animals developed unmistakable symptoms of chronic pneumonia they were killed for study of the gross lesions, histological and bacteriological examinations, and for carrying on transmission experiments.

The sheep were killed by bleeding from the carotid artery and jugular vein. Care was taken not to injure the trachea and thereby permit the inspiration of blood or extraneous contamination. Some of the animals in the advanced stages of the disease were killed shortly after their arrival, and others in which the symptoms were less marked were held for various periods before slaughter. Accordingly, examinations were made of subjects in which the symptoms and lesions varied from slight to those of the advanced type, in which the animals exhibited what was considered to be the last stages of the disease.

At the time gross-lesion examinations were made, small portions of the diseased lung tissue and bronchial and mediastinal lymph glands

[3] This station, now known as Animal Disease Station, has been moved to and made a part of the National Agricultural Research Center, Beltsville, Md.

were placed in fixing solution for later histological examination. Bacteriological examination was made of the lung tissue and associated lymph glands.

Immediately after removal of the lungs from the diseased sheep, the pneumonic areas, including also portions of the lung at the juncture of the diseased and apparently healthy tissue, together with the associated lymph glands, were cultured for aerobic and anaerobic organisms. Aerobic cultures were made by spreading the diseased tissues over the surface of ordinary agar medium and agar medium to which 5 percent of blood serum was added. Anaerobic cultures were made by placing cubes of tissue in depths of 0.1 percent of agar, in Smith fermentation tubes of beef-liver infusion broth containing 1 percent of dextrose, and in tubes of beef infusion broth containing finely divided pieces of beef, the medium being covered with neutral paraffin oil. When micro-organisms were recovered from lung specimens, antigens were prepared from them and agglutination tests and complement-fixation tests were made with blood serum from the host to determine whether specific antibodies were present.

Transmission experiments were made by exposure of healthy sheep to the following materials from diseased sheep: Cultures of organisms from the lung, fresh blood, puslike lung exudate, and emulsified lung tissue, the last-mentioned frequently including portions of the mediastinal lymph gland. Exposure to these materials was made by feeding and by subcutaneous, intravenous, intratracheal, and intrapulmonary inoculations. Contact exposures were also made by placing healthy sheep in pens with the diseased sheep for periods ranging from 6 to 12 months. The exposed sheep were held under observation for varying periods. In the earlier experiments the animals were disposed of in from 3 to 6 months, but in the later experiments most of the sheep were held from 6 to 12 months before slaughter for post-mortem examination. At least 18 of the animals were kept between 11 and 12 months after exposure.

DATA OBTAINED FROM SHEEP NATURALLY AFFECTED WITH CHRONIC PROGRESSIVE PNEUMONIA

GROSS LESIONS

In making the studies of the gross lesions, it was observed that when the thoracic cavity was opened the lungs did not collapse. There was more or less fluid in the cavity. Extensive pleuritic adhesions were frequently present, and in some cases, as a result of these fibrous proliferations, the lungs appeared constricted or lobulated. There was solidification of the affected lungs, varying in extent from partial to almost complete solidification. The observations of the writers indicated that the solidification of the lung tissue has its beginning most frequently near the borders of the diaphragmatic lobes. The consolidated portion of the lungs was usually of a characteristic grayish color, with prominence of the lung lobules on gross sectioning. Small bronchial concretions were sometimes observed. In some cases a puslike material could be squeezed from the bronchi, bronchioles, and alveoli. The bronchial and mediastinal lymph glands usually were swollen and edematous. In all cases examined the lesions were confined to the thoracic cavity.

HISTOPATHOLOGICAL FINDINGS

The usual histological picture was that of a chronic catarrhal pneumonia. The walls of the alveoli showed infiltrations of lymphocytes and large mononuclear cells. The cellular exudate in the alveoli consisted of large mononuclear cells and leucocytes occasionally mixed with red cells. The large mononuclear cells in the lumen of the alveoli were frequently found to be greatly swollen and vacuolated (fig. 1, *A*).

Scattered through the affected lung tissue were frequently seen accumulations of lymphocytes, or lymphoid nodules (fig. 2, *A*), which have been referred to by Marsh (*5*) as "tuberclelike nodules." These nodules were found in the peribronchial and also in the interalveolar tissue. In the consolidated portions of the lung tissue most of the alveoli were filled either with the cellular exudate or with mucous plugs. In some cases many of the alveoli and bronchioles were filled with polymorphonuclear leucocytes.

Epithelial proliferations of the alveoli and bronchioles, which are considered characteristic of progressive pneumonia, were observed in practically all cases of the disease examined by the writers (fig. 3). As the disease progressed there was a gradual increase in the fibrous changes in the areas involved, and in the later stages practically complete fibrosis of a considerable portion of the lung tissue was observed in some cases (fig. 4).

The gross pathology and histopathology of chronic progressive pneumonia, as determined by the writers, were essentially the same as the findings of Marsh (*5*) and Cowdry and Marsh (*3*).

BACTERIOLOGICAL RESULTS

On bacteriological examination, a number of the lung specimens from the sheep naturally affected with chronic progressive pneumonia yielded no growths of micro-organisms, either aerobically or anaerobically. From some specimens there were obtained pure cultures of *Pasteurella oviseptica*, *Corynebacterium ovis*, fungi, or organisms belonging to the diphtheroid group. Other specimens yielded mixed growths of organisms. In no instance, however, was a positive agglutination or complement-fixation reaction obtainable with antigens made from any of the recovered micro-organisms and the blood serum of the host. Thus, the bacteriological examination of sheep affected with chronic progressive pneumonia gave negative results, no micro-organisms having been found that held any promise of being an etiological factor in the disease.

RESULTS OF TRANSMISSION EXPERIMENTS WITH HEALTHY SHEEP

Table 1 shows that visible evidence of pneumonia was found in 21 of the 96 healthy sheep exposed. In this group, the lesions varied in extent from rather slight involvement of the lung tissue to well-marked areas of chronic pneumonia.

Subsequent histological examinations of the lung tissue of the 21 sheep revealed that the pneumonia in 7 was very slight, or atypical, in character. In 10, the lesions were found to be typical of verminous pneumonia; Cowdry (*2*) has called attention to the fact that verminous pneumonia in some cases may simulate the lesions of chronic progressive pneumonia. In the remaining four sheep, nos.

781, 813, 820, and 852, there were well-marked lesions suggestive of chronic progressive pneumonia, and in these cases no nematodes or nematode eggs were found. In view of the fact, therefore, that the

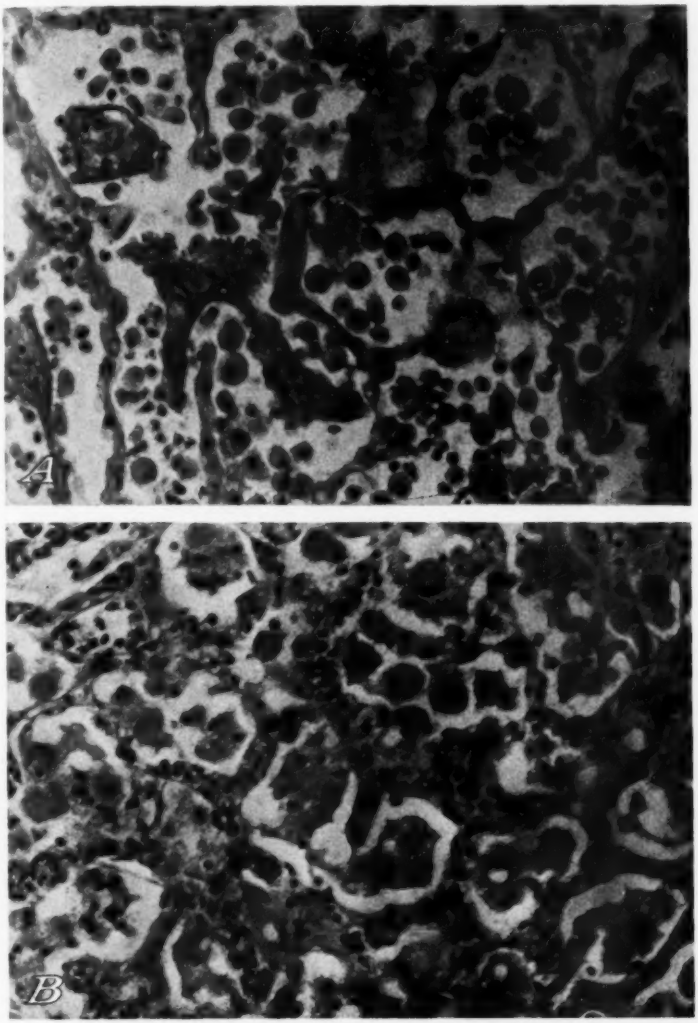

FIGURE 1.—*A*, Section of lung of sheep 693, naturally affected with chronic progressive pneumonia. Note extensive cellular infiltration of alveoli and interalveolar tissue, the former consisting chiefly of the large mononuclear cells. × 400. *B*, Section of lung of sheep 781, which was inoculated with lung material from sheep 777, naturally affected with the disease. Note similarity with figure 1, *A*, in the thickening of alveolar walls and cellular infiltration of alveoli and interalveolar tissue. × 400.

results of exposure were apparently negative for all the experimentally exposed sheep except those just referred to, detailed information is given only for these four.

TABLE 1.—*Results of tests with sheep exposed to chronic progressive pneumonia by various methods*

Healthy sheep exposed (number)	Material used in exposure tests [1]	Method of exposure [2]	Incidence of pulmonary lesions found on post-mortem examination			
			Sheep with no lesions	Sheep with parasitic lesions	Sheep with slight pneumonic lesions	Sheep with lesions suggestive of chronic progressive pneumonia
			Number	*Number*	*Number*	*Number*
22	Emulsified lung tissue from naturally affected sheep.	Intrapulmonary inoculation.	[1] 13	4	2	3
11	do	Intravenous inoculation.	[3] 7		3	1
3	do	Intratracheal inoculation.	2	1		
9	Organisms from lung of naturally affected sheep.	Intrapulmonary inoculation.	[3] 5	2	2	
8	do	Intravenous inoculation.	[3] 6	2		
3	do	Intratracheal inoculation.	3			
9	do	Feeding	9			
2	Fresh blood from naturally affected sheep.	Intravenous inoculation.	2			
15	None	Contact	15			
11	Emulsified lung tissue from experimentally exposed sheep.	Intrapulmonary inoculation.	10	1		
1	do	Intravenous inoculation.	1			
1	Puslike material from lung of experimentally exposed sheep.	Subcutaneous inoculation.	1			
1	Organisms from lung of experimentally exposed sheep.	Intravenous inoculation.	1			

[1] In the case of the emulsified lung tissue, small portions of the mediastinal lymph gland were usually included.

[2] The desirability of arranging the experimentally exposed sheep in the various groups precludes showing the time elapsing between exposure and post-mortem examination of the individual animals, which varied rom 3 to 12 months.

[3] 1 of these sheep died from acute septic infection shortly after inoculation.

The four sheep were inoculated with emulsified lung tissue, including a small amount of mediastinal lymph gland, from four of the diseased sheep. Exposure was made by the intrapulmonary method except in the case of sheep 813, which was exposed intravenously. The time elapsing from inoculation to slaughter was as follows: 8 months 4 days for sheep 781, 11 months 1 day for sheep 813, 9 months 4 days for sheep 820, and 11 months 22 days for sheep 852. None of these animals showed any definite clinical symptoms at the time of slaughter. The gross lesions found in each of the four animals were as follows:

In sheep 781, both lungs contained numerous grayish, solidified areas throughout, and, in addition, one larger area, approximately 2 inches in diameter, was observed in the postero-superior portion of the right lung.

In sheep 813, chronic pneumonic areas more or less limited in character were found in both lungs.

In sheep 820, when the thoracic cavity was opened the lungs did not collapse. Numerous adhesions were noted. The lungs contained

numerous grayish consolidated areas with more uniform solidification of the lung tissue toward the borders of the diaphragmatic lobes.

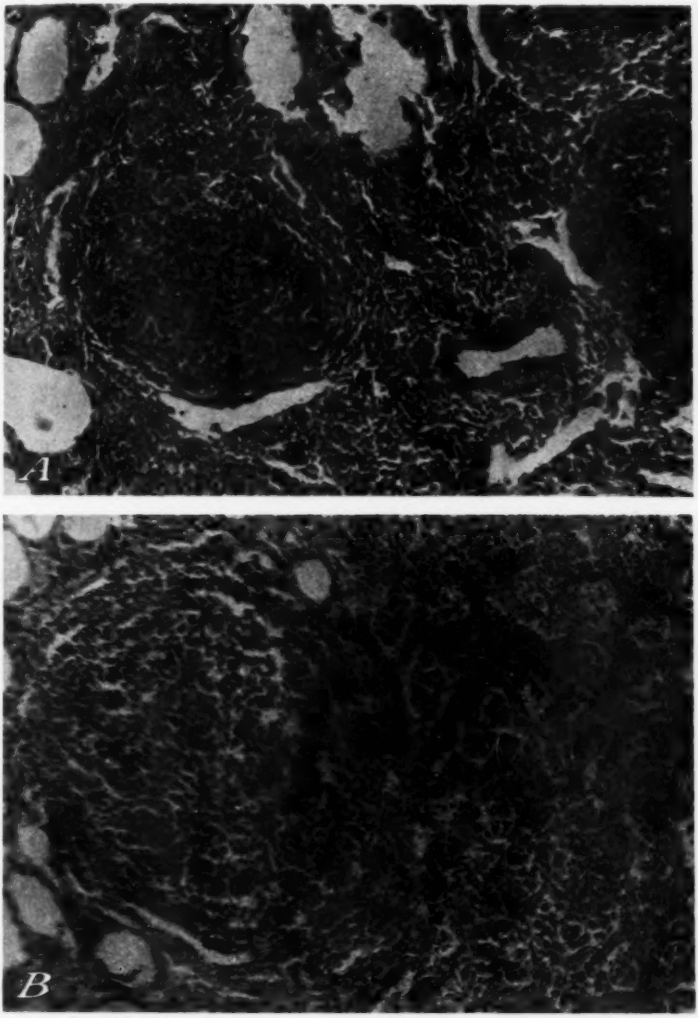

FIGURE 2.—*A*, Section of lung of sheep 712, naturally affected with chronic progressive pneumonia. Note typical interalveolar lymphoid deposits. × 120. *B*, Section of lung of sheep 852, which was inoculated with lung material from sheep 827, naturally affected with the disease. Note similarity with figure 2, *A*, particularly with respect to the lymphoid deposits. × 120.

On gross section the solidified areas closely simulated the lesions of chronic progressive pneumonia.

In sheep 852, when the thoracic cavity was opened the lungs did not collapse. There were some constrictions or lobulations as a

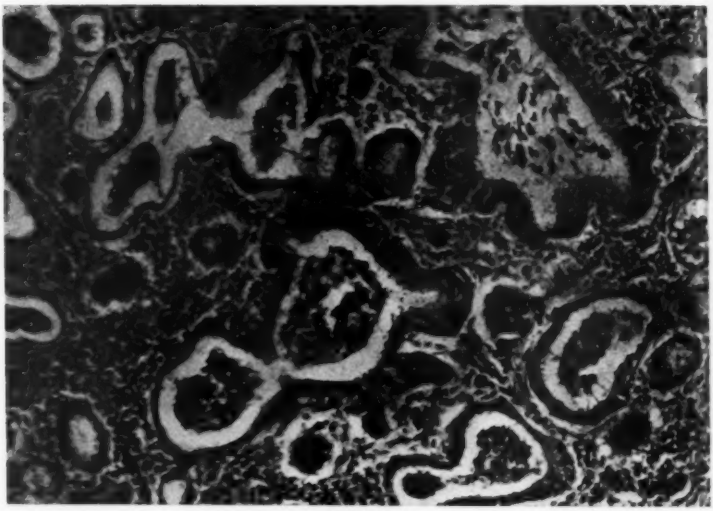

FIGURE 3.—Section of lung of sheep 728, naturally affected with chronic progressive pneumonia. Note typical bronchial proliferation. × 120.

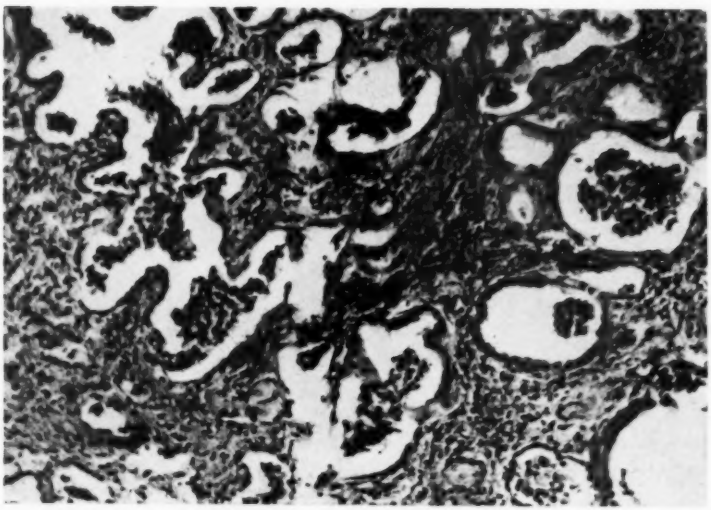

FIGURE 4.—Section of lung of sheep 797, naturally affected with chronic progressive pneumonia and in the later stages of the disease. Note the rather extensive fibrosis of the lung tissue. × 120.

result of fibrous proliferations. Numerous grayish solidified areas were seen throughout both lungs. Solidification was more uniform toward the superior portion and borders of the diaphragmatic lobes. The bronchial and mediastinal lymph glands appeared to be enlarged and edematous.

The bacteriological results from these four sheep were considered negative from the standpoint of pathogenic micro-organisms. Culture media inoculated from the lung tissue of one of the sheep, no. 813, remained sterile. The lung tissue of the other three yielded a limited number of organisms, none of which was found to be pathogenic.

Although there were slight variations, on the whole the pathological changes observed in the lungs of sheep 781, 813, 820, and 852, were similar. There was thickening of the alveolar walls as a result of the interalveolar cellular infiltrations. There was also cellular exudate into the alveoli, consisting mostly of the large mononuclear cells and lymphocytes. Note the similarity, in these respects, between the lung tissue of sheep 781 (fig. 1, *B*), and that of a sheep 693, naturally affected with chronic progressive pneumonia (fig. 1, *A*).

In the lung tissue of one of the sheep, no. 852, there were also rather extensive infiltrations of polymorphonuclear leucocytes. Occasional localized capillary engorgement was noted. Typical peribronchial and interalveolar lymphoid nodules were a prominent feature of the lesions in all four animals, as illustrated for sheep 852 (fig. 2, *B*). In this respect also, attention is called to the similarity between the section of lung of this sheep and that of a naturally affected sheep (fig. 2, *A*).

Epithelial proliferations of the alveoli and bronchioles were also noted, those in sheep 820 (fig. 5, *B*) being similar to those in one of the naturally affected sheep (fig. 5, *A*). These proliferations were somewhat more marked in sheep 852 (fig. 6) than in the others. In this sheep also more extensive fibrous changes were noted in the lung tissue than in any one of the other three.

Five healthy sheep approximately 1 year of age were inoculated by the intrapulmonary method with material from the lung and mediastinal lymph gland of three of the experimental sheep as follows: Two animals from sheep 852, two from sheep 820, and one from sheep 781. No inoculations were made from sheep 813. One animal died approximately 1 month after inoculation, one was held for 3 months, one for 6 months, one for 11 months, and one for 12 months. None of the five animals showed visible evidence of chronic progressive pneumonia on post-mortem examination.

DISCUSSION

Although a rather large number of transmission experiments were made, lesions suggestive of chronic progressive pneumonia were found in only 4 of the 96 experimentally exposed sheep. The writers believe that the lesions in these four sheep resulted from experimental exposure. Conclusive evidence of direct transmission was lacking, however, because subsequent attempts to produce similar lesions in other sheep by inoculating them with lung material from three of the four animals in question gave negative results. Similar difficulty has been experienced by other investigators of this disease. This may have been due to not having affected sheep at the proper stage

for transmission or to a lack of susceptibility in the normal sheep that were exposed to the disease. That there is a lack of susceptibility in a large percentage of sheep seems to be evidenced by the fact that

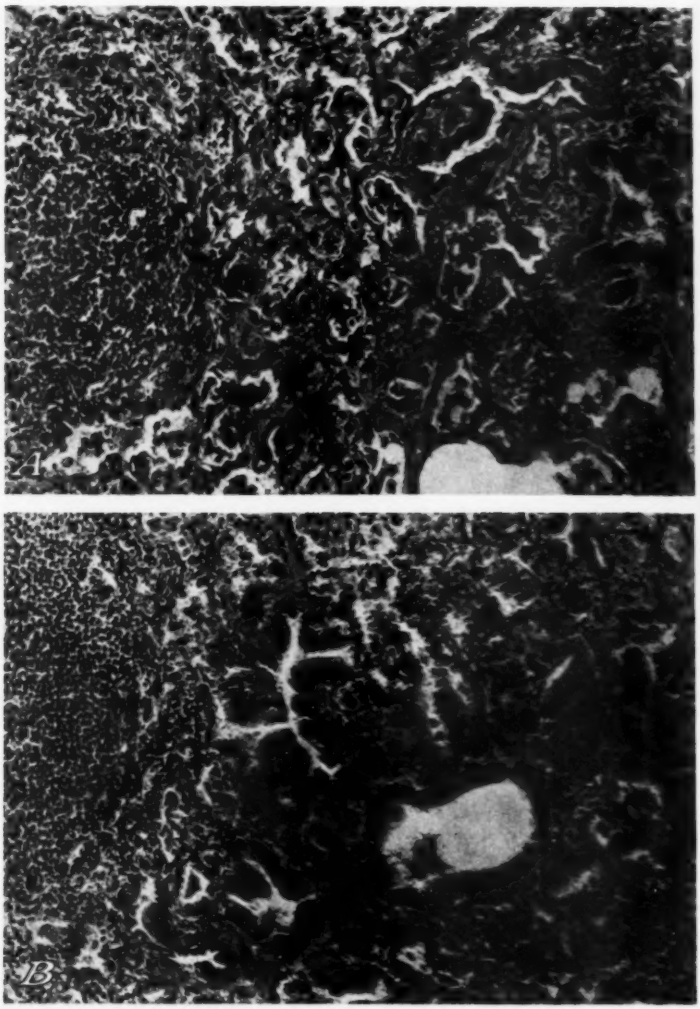

FIGURE 5.—*A*, Section of lung of sheep 829, naturally affected with chronic progressive pneumonia. Sheep 813 was inoculated with lung material from this animal. × 120. *B*, Section of lung of sheep 820, which was inoculated with lung tissue from sheep 830, naturally affected with chronic progressive pneumonia. × 120. Note similiarity of lesions to those in lung of sheep 829 (fig. 5, *A*), particularly with regard to proliferation of bronchioles and lymphoid nodules.

only from 2 to 10 percent of the animals become affected in herds in which the disease is found. In the present experiments, the difficulty in transmission might also be accounted for by the difference

in environment at the experiment station where the transmission experiments were conducted and that of the areas where the disease occurs naturally. In the latter environment there may be predisposing influences as a result of which sheep become more susceptible, and there also seems to be a possibility that recovery may take place in some cases when the animals are removed from such environmental conditions.

Cowdry and Marsh (*3*) are of the opinion that there is some evidence that animals in the earliest stages of the disease may recover. In the present investigation three sheep naturally affected with the disease showed only slight evidence of it when they were slaughtered. One of these sheep was slaughtered after being held under observation

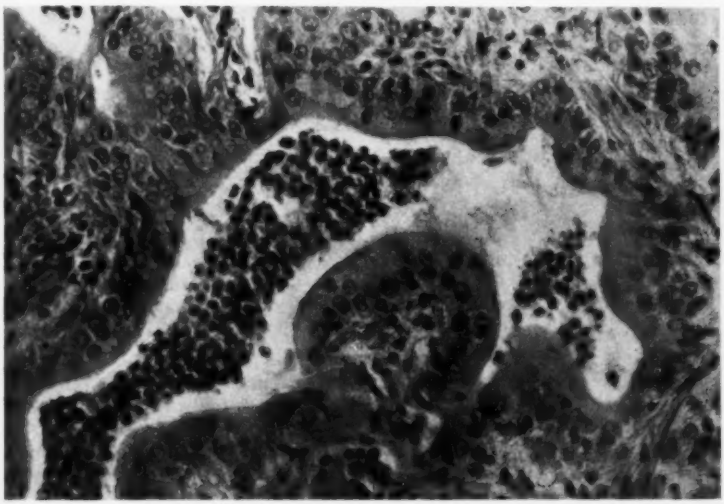

FIGURE 6.—Section of lung of sheep 852, which was inoculated with lung material from sheep 827, naturally affected with chronic progressive pneumonia. Note marked proliferation of the bronchial epithelium. × 400.

for 9 months, and the other two after a period of 18 months. Although no well-marked symptoms were noted in these animals during the period of observation, it is presumed that some clinical evidence of the disease had been observed prior to shipment from Montana; and if so these cases strongly suggest the possibility of recovery, particularly if affected sheep are removed from their original environment during the early stages of the disease.

Records on the length of time that diseased sheep survive after being removed from their native environment show individual variation in this respect. Two of the sheep from Montana were kept at the Experiment Station at Bethesda, Md., for more than 18 months. One of these showed little clinical evidence of the disease when it was slaughtered, and on post-mortem examination the pneumonic lesions were found to be somewhat limited in extent. In the other sheep there was some evidence of difficult respiration, and somewhat more advanced lung lesions were found on post-mortem examination.

The authors were unable to determine whether one or both of these may have been arrested cases of the disease or how long they would have survived if they had not been slaughtered at the conclusion of the experiments. According to the observations of the authors in a limited number of cases, sheep in which the clinical symptoms are well marked usually become gradually worse, but may survive for 6 months or longer.

The character of the lesions found in chronic pneumonia have been of particular interest to investigators. The very pronounced epithelial proliferations, one of the very characteristic changes found in chronic progressive pneumonia and jagziekte, have provoked considerable discussion as to the cause of such changes. Cowdry (*2*) offers the suggestion that such epithelial proliferations, which also occur occasionally in other types of pneumonia in sheep, may indicate that the pulmonary epithelium of sheep is peculiarly unstable, being unusually susceptible to growth stimuli or to influences which tend to invalidate normal inhibitory mechanisms. De Kock (*4*) concluded from his studies of the lung lesions in jagziekte that the adenomalike proliferations of the epithelium in such cases were probably neoplastic in nature. It must be admitted that these proliferations of the lung epithelium, in some cases, assume an adenomatous appearance strongly suggestive of neoplastic changes.

SUMMARY

In investigations relating to chronic progressive pneumonia of sheep, particularly its etiology and transmission, 23 diseased sheep from Montana and 96 healthy sheep from Maryland were used in experiments, most of which were carried on at Bethesda, Md.

On bacteriological examination, lung specimens from a number of sheep affected with chronic progressive pneumonia failed to yield growths of organisms of any kind. From other specimens different types of organisms were isolated, none of which could be demonstrated, through exposure tests or otherwise, to be a possible etiological factor.

In the diseased sheep the gross lesions and histopathological findings, which are of the nature of a chronic catarrhal pneumonia, are essentially the same as have been described by other investigators for this disease and also for jagziekte.

Because of the similarity of the gross and histological lesions found in 4 of the 96 experimentally exposed sheep to those found in naturally occurring chronic progressive pneumonia, it is the opinion of the writers that the lesions in these four sheep were the result of experimental exposure. Conclusive evidence to this effect is lacking, however, because of the inability to reproduce the typical lesions by further inoculation of the diseased tissues found in these four sheep.

These experimental studies of chronic progressive pneumonia of sheep have failed to reveal any specific etiological factor of bacterial nature or otherwise.

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DETERMINATION OF THE LESS REFINED MINERAL OILS ON LEAF SURFACES AFTER SPRAYING [1]

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INTRODUCTION

It was shown in a previous paper (*3*) [2] that small quantities of a refined mineral oil retained by leaves after spraying could be quantitatively recovered from the leaf surfaces of camphor-trees and on pecan and Satsuma orange trees. The determinations were made with a single oil, known as Marcol. Success in the later steps of the procedure depended upon the fact that concentrated nitric acid was shown to have no appreciable action on Marcol. Treatment of the samples with this reagent was one of the important steps, and the procedure, although possessing utility for refined oils, was not claimed to be applicable to unrefined oils, since the extent of the action of the nitric acid upon this type was not demonstrated. A suitable procedure for the estimation of the less refined oils on different species of foliage has since been worked out, and is described in this paper.

OILS STUDIED

A number of the less refined oils differing considerably from one another, and such as might be used in dormant spraying, were selected for study of the accuracy obtainable in analyses. Certain physical and chemical constants of these oils are given in table 1.

TABLE 1.—*Properties of mineral oils employed in determinations and of the highly refined Marcol used in previous experiments*

Oil	Color	Cloud test [1]	Pour test [1]	Specific gravity, 20°/20° C.	Saybolt viscosity at 37.8° C.	Volatility [3]	Unsulphonated residue [2]
		° F.	*° F.*		*Seconds*	*Percent*	*Percent*
Cayuga	Yellow	48	20	0.8841	102	1.16	62.0
Osage	do	56	35	.8898	140	.74	58.4
Red Engine	do	40	35	.8939	154	.15	56.0
L-1780	do			.8947	220	[4] 1.00	72.9
Alcopol	do	2	−10	.9188	221	1.74	59.6
Niantic	do	46	40	.8042	307	.25	[5]
White Rose	Water white			.8512	95	[4] 1.44	94.0
Marcol	do	58	30	.8419	86	1.58	96.8

[1] Determined according to the American Society for Testing Materials Method D99-22T (*7, pp. 32-36*). In the description of this method temperatures are given in degrees Fahrenheit.
[2] Determined according to method adopted by the Insecticide Division, but at 110° C. See Wiley (*8, v. 2, p. 566*).
[3] Determined according to method adopted by the Association of Official Agricultural Chemists (*1*).
[4] Volatility determined over 24 hours at 100° F. (37.8° C.), see Dawsey (*2*).
[5] Large amount of free carbon prevented reading the oil column after test on this oil.

The object in listing these constants is to furnish a description of the materials employed rather than to supply any data directly useful in calculating the quantity of oil on foliage. For example, the constants do not afford information concerning the oxidation, nitration, or splitting of unsaturated, aromatic, or naphthalenic compounds which may be components of the unrefined oils before treatment with nitric acid.

[1] Received for publication Dec. 12, 1935; issued June 1936.
[2] Reference is made by number (italic) to Literature Cited, p. 693.

RECOVERY OF OILS FROM CAMPHOR-TREE LEAVES

DETERMINATION OF OPTIMUM NITRATION PERIOD

Experiments were first performed to determine the extent of the action of nitric acid upon some of the oils under the analytical conditions previously adopted. Up to the addition of nitric acid the procedure was substantially the same as that described for the recovery of refined oil from leaves of the camphor-tree (*Cinnamomum camphora*) (*3*). Each sample contained 100 leaf disks 30 mm in diameter, to which weighed quantities of oil had been added. After extraction the waxes were frozen out, the filtrates transferred to Babcock bottles, and the ether-alcohol solutions evaporated, so that the residues consisted only of mineral oil, natural leaf oil, and chlorophyll. The acid treatment, with which this paper is chiefly concerned, consisted in adding 6.0 cc of concentrated nitric acid to the cold oil residues in the Babcock bottles and heating the bottles in boiling water for a chosen length of time with agitation at 5-minute intervals. Since the quantity of oil present was rarely more than 100 mg, nitric acid was always present in large excess.

The volume of 6.0 cc nitric acid was chosen because of the construction of the capillary bottles. The inlet tubes on all bottles projected about two-thirds of the distance down into the lower bulbs, so that a greater volume of acid would have caused loss of the contents during the evolution of the nitrogen oxides which accompanied the oxidation. It was necessary to carry out the oxidation at high temperatures to eliminate completely the leaf materials extracted with the oil.

Table 2 shows the weights and percentages of the various unrefined petroleum oils that were undestroyed by the acid under the conditions of the experiment. In figure 1, which gives these results graphically, it may be seen that the nitric acid did not attack any two of the oils to exactly the same degree. These curves were employed in estimating the optimum heating period for each oil, so that minimum errors would be introduced into the analyses of the respective oils. For example, for Cayuga, Osage, Red Engine, Alcopol, and Niantic oils, the slopes were least after heating for 60, 60, 60, 20, and 15 minutes, respectively. In the interest of shortening the procedure, however, the following nitration periods were adopted for the respective oils: 30, 50, 30, 30, and 15 minutes.

CHECK ANALYSES

On the basis of these estimates of the proper nitration period for each oil, a number of check analyses were carried out to establish the reliability of such methods in the determination of unknown samples. Data from series of such check analyses on four of the less refined oils are listed in table 3. Each was run a different time, and no attempt was made to maintain a consistent strength of nitric acid from day to day. It is therefore highly probable that no two oils were treated with acid of exactly the same concentration, although all the samples of each oil were treated with acid of the same strength.

TABLE 2.—*Recovery of unrefined petroleum oils after treatment with nitric acid for various periods of time in the presence of materials extracted from camphor-tree leaves*

Oil and sample no.	Nitration period	Oil added	Oil recovered	
	Minutes	*Milligrams*	*Milligrams*	*Percent*
Cayuga:				
1	15	97.3	87.9	90.3
2	30	81.4	68.8	84.5
3	45	99.1	80.2	80.9
4	60	109.6	85.4	77.9
Osage:				
1	15	93.8	81.6	87.0
2	30	86.0	71.1	82.7
3	45	90.9	72.3	79.5
4	60	89.4	71.5	80.0
Red Engine:[1]				
1	15	96.7	95.0	98.2
2	30	118.1	114.4	96.9
3	45	98.9	93.9	94.9
4	60	82.4	77.7	94.3
Alcopol:				
1	15	88.4	72.4	81.9
2	30	114.0	88.5	77.6
3	45	93.1	60.5	65.0
4	60	96.4	52.9	54.9
Niantic:				
1	15	81.5	63.6	78.0
2	30	99.3	74.7	75.2
3	45	80.5	63.3	78.6
4	60	95.4	74.3	77.9

[1] The concentration of the nitric acid used with these samples was unknown, but its specific gravity was considerably less than 1.42 because the acid was exposed to the sunlight in an open beaker for several days before use. Compare sample 2 with samples of the same oil in table 3, where stronger acid was employed.

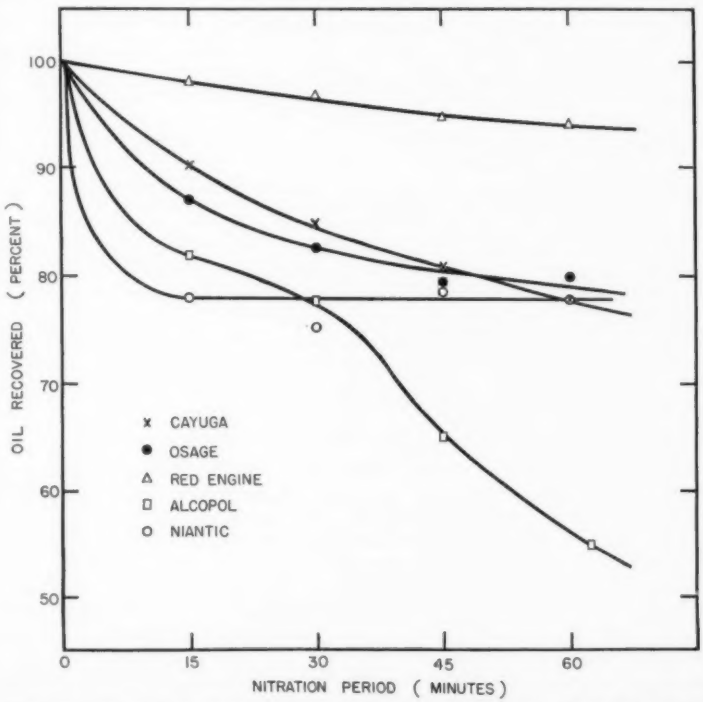

FIGURE 1.—Percentages of oils recovered after treatment of samples with nitric acid for different lengths of time. Plotted from the data in table 2.

TABLE 3.—*Check analyses on four unrefined mineral oils in the presence of materials extracted from camphor-tree leaves, showing the agreement between the calculated oil and that actually present*

Oil and sample no.	Nitration period	Oil added	Oil recovered		Calculated oil added	Error
	Minutes	*Milligrams*	*Milligrams*	*Percent*	*Milligrams*	*Percent*
Cayuga	30					
1		72.9	61.5	84.4	72.8	−0.1
2		107.2	90.6	84.5	107.2	0
3		81.4	68.8	84.5	81.4	0
4		69.6	59.0	84.8	69.6	+.4
5		94.1	79.5	84.5	94.1	0
6		81.4	68.5	84.2	81.1	−.4
7		57.9	48.8	84.3	57.8	−.2
Mean				84.5		
Osage	50					
1		89.6	71.2	79.5	90.4	+.9
2		89.7	69.4	77.4	88.1	−1.8
3		88.8	69.2	77.9	87.8	−1.1
4		89.4	69.9	78.2	88.7	−.8
5		90.9	72.3	79.5	91.8	+1.0
6		84.8	68.3	80.5	86.7	+2.2
Mean				78.8		
Red Engine[1]	30					
1		116.6	99.0	84.9	117.0	+.3
2		79.8	67.3	84.3	79.5	−.4
3		98.8	83.4	84.4	98.6	−.2
4		74.7	63.2	84.6	74.7	0
5		115.0	97.7	85.0	115.5	+.4
6		104.9	88.4	84.3	104.5	−.4
7		74.8	63.2	84.5	74.7	−.1
8		100.3	85.0	84.7	100.5	+.2
Mean				84.6		
Niantic	15					
1		79.4	63.8	80.4	79.9	+.6
2		99.1	78.6	79.3	98.5	−.6
3		95.2	76.1	79.9	95.4	+.2
4		94.9	75.4	79.5	94.5	−.4
Mean				79.8		

[1] The percentage of Red Engine oil destroyed was considerably larger than that in the similar sample 2, able 2. This is accounted for by the fact that stronger acid was employed here.

The data show that the percentages of oil undestroyed were substantially the same in different samples of the same oil. It was concluded, therefore, that it would be practical to determine unknown quantities of oil on sprayed trees simply by extracting check samples containing known quantities of oil and equal numbers of leaf disks along with the corresponding unknown samples, and, after calculating the proper correction factor, to apply it to the unknowns treated under identical conditions.

ALTERNATIVE METHOD

The Alcopol oil was reserved for the purpose of demonstrating an alternative method of estimating such oils in the presence of extracts of camphor-tree leaves. After the optimum nitration period of 30 minutes had been selected, several tests were run with this oil to determine what strength of acid would be required to destroy all the residual leaf materials and yet not attack the petroleum oil. A series of such tests is recorded in table 4 (samples 1 to 4). The concentration of 4 cc of nitric acid to 2 cc of water proved to be the optimum for the apparent elimination of the leaf materials, and samples 5 to 8 were run with this strength of acid to determine the reliability of this treatment. The agreement of the data in the table show that it is possible to recover completely unrefined oils on camphor-tree foliage,

provided the proper acid concentration is first determined for the specific oil and definite numbers of disks. There is a possibility, however, that the traces of undestroyed leaf oil just compensated for the petroleum oil taken up by the diluted nitric acid.

TABLE 4.—*Check analyses of Alcopol oil, showing the error introduced by nitration at various concentrations of acid*

Sample no. [1]	Nitric acid added	Water added	Oil added	Oil recovered	
	Cubic centimeters	*Cubic centimeters*	*Milligrams*	*Milligrams*	*Percent*
1	6	0	114.0	88.5	77.6
2	5	1	98.5	90.3	91.7
3	4	2	96.1	96.0	99.9
4	3	3	95.6	100.9	105.5
5	4	2	94.2	94.1	99.9
6	4	2	99.0	98.1	99.1
7	4	2	97.5	96.2	98.7
8	4	2	101.1	100.5	99.4

[1] Samples 1 to 4 were trial samples. Samples 5 to 8 show the results obtained under the optimum acid concentration.

RECOVERY OF OILS FROM SATSUMA ORANGE LEAVES

The recovery of two of the oils was also studied in the presence of extracts of the leaf of Satsuma orange (*Citrus nobilis unshiu*), with the adoption of an optimum nitration period as in the experiments with camphor-tree leaves. In these experiments the natural waxes were frozen out from the initial extracts under the previously determined optimum conditions. The results in table 5 show that a correction factor is applicable in the determination of these less refined oils on Satsuma orange foliage. The error involved, however, although within ±4 percent, was larger than in the case of the camphor-tree leaves, and also slightly larger than that obtained with the more highly refined Marcol when extracted with orange leaves, as reported in the earlier paper (*3*).

TABLE 5.—*Check analyses of Red Engine and Cayuga oils in the presence of extracts of Satsuma orange leaf*

Oil and sample no.	Oil added	Oil recovered		Calculated oil added	Error
Red Engine:	*Milligrams*	*Milligrams*	*Percent*	*Milligrams*	*Percent*
1	112.7	79.5	70.5	111.6	−1.0
2	116.1	85.4	73.6	119.9	+3.3
3	78.5	55.3	70.4	77.6	−1.1
4	75.5	54.6	72.3	76.7	+1.6
5	93.7	66.2	70.7	92.9	−.9
6	93.1	65.1	69.9	91.4	−1.8
Mean			71.2		
Cayuga:					
1	108.5	72.9	67.2	110.7	+2.0
2	89.5	58.1	64.9	88.3	−1.3
3	114.8	76.7	66.8	116.5	+1.5
4	62.1	39.4	63.4	59.8	−3.7
5	84.7	55.3	65.3	84.0	−.8
6	104.9	70.6	67.3	107.2	+2.2
Mean			65.8		

RECOVERY OF OILS FROM CHRYSANTHEMUM LEAVES

Certain variations and improvements were introduced during the development of a suitable method for the determination of petroleum oils on chrysanthemum foliage. For these tests leaves from the Old

Rose variety of chrysanthemum (*Chrysanthemum hortorum*) plants were used. The potted plants were reared in a greenhouse and were of uniform size and age. Before extraction, samples contained 200 leaf disks 1 cm in diameter together with definite quantities of petroleum oil.

EFFICIENCY OF EXTRACTION

Since the oil absorbed in the chrysanthemum leaf disks was to be extracted with ethyl ether, it was necessary to ascertain the efficiency of extraction before making determinations of the petroleum oil from the plants.

In one experiment a separate set of eight plants was sprayed with a 2-percent emulsion prepared from the highly refined White Rose oil with bone glue as emulsifier. After the water from the spray had dried, four replicate samples of leaf disks were cut and allowed to stand 2 days for the oil to penetrate the leaf tissue, thus making it harder to wash the oil from the disks. They were then extracted in several ways to determine the quantities of combined oil and wax recoverable during each separate and successive washing.

The results showed that it was impractical to wash the disks more than four times because of the time consumed, but that at least 30 to 50 cc of ether should be employed each time. For example, one sample, in five successive washings with 40 cc of ether, gave 0.0942, 0.0239, 0.0056, 0.0019, and 0.0005 cc of combined oil and wax. Proportionately larger quantities of oil were taken off in the first washing with larger volumes of ether. It was also found that the longer the disks soaked the larger were the quantities of oil removed in the initial washings. The first four washings thus recovered substantially all the quickly soluble material in the samples. In all the following reported determinations the samples of chrysanthemum disks were washed four times with 35 cc of ether.

In other experiments, where approximately 24 hours elapsed between the spray application and the leaf extraction, from 90 to 95 percent of the total deposit was recovered. These tests were carried out by extracting four times with 35 cc of ether, measuring the oil so obtained, drying and grinding the partially exhausted leaf disks, washing the powdered material repeatedly for 2 hours in an extraction apparatus, and measuring and calculating the spray oil left in the leaves after the first extractions.

TESTS ON OMISSION OF WAX-FREEZING STEP IN PROCEDURE

Experiments were then made to ascertain whether the wax-freezing step might be omitted from the procedures employed in the studies with camphor-tree and orange leaves. It was found possible, instead of separating the wax from the petroleum oil, to measure the two together and then calculate the oil by subtraction.

A set of tests was conducted to determine the quantity of extractable wax in samples of 200 leaf disks to which known weights of White Rose petroleum oil were added. These tests consisted in filtering the ether extracts, evaporating off the solvent, transferring the residue to calibrated Babcock bottles and treating it with 5 cc of nitric acid at 100° C., and then measuring the combined oil and wax in the capillaries after filling with more nitric acid and centrifuging. It was necessary to centrifuge at about 50° because there was sufficient wax present to solidify the oil in the tubes at room temperature. The data in table 6 show that the quantity of extractable wax, in the case

of chrysanthemum leaves, is independent of the petroleum-oil content and remains constant within workable limits.

TABLE 6.—*Extractable wax in samples of chrysanthemum leaf disks to which known weights of White Rose petroleum oil were added*

Sample no.	Oil added	Oil and wax recovered	Wax, by difference
	Milligrams	*Milligrams*	*Milligrams*
1	17.5	22.0	4.5
2	29.8	34.2	4.4
3	38.0	42.2	4.2
4	46.6	50.6	4.0
5	56.1	60.8	4.7
6	67.0	71.9	4.9
7	71.3	75.8	4.5
8	81.3	85.4	4.1
Mean			4.4

Since it has been shown that the extractable wax was completely removed under the stated conditions, it follows, for samples of lesser wax content at least, that the quantity of recoverable wax would be proportional to the number of leaf disks treated.

CHECK ANALYSES

After the establishment of the proper conditions under which to extract chrysanthemum leaves, such that substantially complete recovery of the insecticidally active portions of mineral oil might be accomplished, and it had been shown that the usual procedure might be simplified by omitting the wax-freezing step, a series of five tests was run to determine the degree of accuracy attainable in the estimation of the oil content in check samples. One of the less refined oils, no. L–1780, was chosen for these tests. Table 7 shows the results obtained when 5 cc of nitric acid was added to each sample followed by the usual intermittent agitation and heating. After account had been taken of the corrections for the undestroyed leaf wax present because of the omission of the wax-freezing step, and for the oil destroyed by the acid treatment, the calculated quantities of oil present were found to be within ± 2.5 percent of their true values. It was also shown that the accuracy of the calculated figures was again independent of the actual oil content of the samples.

TABLE 7.—*Check analyses of oil L–1780 in the presence of chrysanthemum leaf material*

Sample no.	Oil added	Oil and wax recovered [1]	Oil recovered [2]		Calculated oil added	Error
	Milligrams	*Milligrams*	*Milligrams*	*Percent*	*Milligrams*	*Percent*
1	50.7	44.6	40.2	79.2	51.7	−2.0
2	65.2	54.8	50.4	77.3	64.8	−.6
3	77.5	63.2	58.8	75.9	75.6	−2.5
4	101.3	83.7	79.3	78.3	101.9	+.6
5	116.8	95.8	91.4	78.3	117.5	+.6
Mean				77.8		

[1] The specific gravity of the oil and wax mixture was assumed to be the same as that of the oil, namely, 0.8947 at 20°/20° C.

[2] The weight of oil recovered was obtained by subtracting from the weight of oil and wax recovered the constant 4.4 mg, obtained for the mean weight of wax recovered in the previous series of experiments with White Rose oil (table 6). It will be noted that this constant agrees well with the figure 3.8 mg, obtained by calculating the most probable values for the weight of wax and the percentage of oil recovered from the data on oil L–1780.

DISCUSSION

There may be several reasons why greater accuracy was not obtained by the methods proposed. Possible errors in calibration of the Babcock bottles undoubtedly offer one explanation. For example, three separate determinations of volume were made for each bottle and the mean of these determinations was calculated. Thus, for one of the bottles the following figures were obtained for volume per millimeter of tube length: 0.0014159, 0.0014397, and 0.0014410 cc. The mean of these figures was 0.0014322 cc. The deviations from the mean are found to be 1.12, 0.56 and 0.63 percent, respectively. Since the error in calibration may be as large as 1.12 percent, determinations might regularly show errors of this size.

The investigations with camphor-tree and Satsuma orange leaves reported in this paper were made at New Orleans, La., while the studies with chrysanthemum leaves were carried out after the field station had been transferred to Wooster, Ohio. Since the omission of the wax-freezing step in the procedure for the chrysanthemum foliage proved to be a simplification without loss of accuracy, it must be considered to be an improvement. It would have been desirable to study the reliability of the methods for the other two species of leaves when this step was eliminated, but since no camphor-tree or orange leaves were available in the northern location such an investigation could not be undertaken. It should be possible, however, to simplify those two procedures also by omitting the freezing step, provided the wax-residue constant is first determined for given numbers of leaf disks, as illustrated in table 7.

Since the appearance of English's original method (*4*), followed by the improvements of Dawsey and Haas (*3*), two other methods for the determination of oil-spray deposit have been proposed. Swain and Green (*6*) have called attention to the distinction between the insecticidally active deposit (surface oil) and the total deposit (surface oil plus that absorbed in the leaf tissue). They have developed a comparatively simple method for an approximate determination of the surface oil, which involves washing leaf surfaces with methylene chloride, evaporating the solvent, and then weighing the residue consisting of plant wax and petroleum oil. According to the published results, the procedure is most applicable to determinations made immediately after the water has evaporated when there has been little time for the oil to penetrate the leaf tissue. Since petroleum oil and leaf-surface wax are known to be mutually soluble, however, uncertainty exists as to exactly what percentage of surface oil can be removed each time by the single methylene chloride washing treatment, particularly in view of the small quantities of the natural wax removed by these investigators in washing unsprayed leaves. Rohrbaugh (*5*) prefers to dry and grind citrus leaves before making extractions with petroleum ether. With the incorporation of certain steps, such as correcting for the quantity of oil destroyed in the sulphuric acid treatment in the case of sulphonatable oils, and correcting for the quantity of natural leaf substances extracted with the spray oil, his method appears to be applicable to the determination of total deposit regardless of the interval between spray application and extraction. All these methods have contributed to the solution of the problem of recovery of oil spray residue from plants.

Judging from what is known in the field at present, in routine work it would seem preferable to adopt procedures capable of giving the total spray deposit, as advocated by Rohrbaugh (*5*), rather than the surface oil, which is in itself a variable quantity dependent upon penetration of the oil into the leaf tissue as influenced by the volatility and time factors. If interest is centered upon the insecticidal action of the oil, it would also appear, from the evidence at hand, that extractions should be made as soon as possible after spraying, in order to minimize the volatilization effect, thereby obtaining definite and reproducible values for the deposit each time.

Two logical criticisms have been made of the general methods employed by English (*4*) and by Dawsey and Haas (*3*): (1) Taking large disks from the center of leaves does not give representative samples, because the oil accumulates principally along the midrib and edges of the leaves; and (2) simply washing the leaves with ethyl ether will not extract the last traces of oil from the leaf tissue. In the method for chrysanthemum leaves given in this paper the first difficulty has been largely eliminated by taking a large number of small disks over the whole leaf area so that advantage of speed in sampling has been retained. With regard to the second criticism, from 90 to 95 percent of the total oil can be recovered, at least with chrysanthemum leaves extracted soon after spraying, by the methods herein described. The procedure, therefore, offers a means for a good approximation to the total oil deposit without recourse to the laborious process of reducing foliage to a powder before extraction, and in addition does not require the use of expensive extraction apparatus.[3] The work is being continued at the Wooster laboratory in a study of extraction methods applicable to vegetable and animal oils as well as to petroleum oils.

SUMMARY

The problem of determination of the less refined petroleum oils in the presence of materials extracted from plant foliage by ethyl ether has been studied. Treatment of the leaf-extract residues containing petroleum with nitric acid under controlled conditions results in destruction of a definite proportion of the oil each time, and by using a predetermined correction factor the original quantity of the oil present can be estimated. Applications are suggested in the determination of oil deposit from the foliage of camphor-tree, Satsuma orange, and chrysanthemum plants following spraying with oil emulsions.

The method of analysis of these sulphonatable oils in the presence of chrysanthemum leaf materials has been simplified, without loss of accuracy, by omitting the usual wax-freezing step as employed in the treatment of the camphor-tree and Satsuma orange leaf extracts.

The efficiency in oil recovery in washing leaf disks taken from sprayed plants with ethyl ether has also been studied. For a limited number of washings recovery is not complete, but from 90 to 95 percent of the oil is removed even when extraction is made 24 hours after the spray application.

[3] Later results have shown that 100 percent of the oil deposit can be recovered by continuous washing with various solvents in an extraction apparatus, provided fresh green leaf disks are used. Drying without powdering prevents complete recovery, however, under the same conditions.

The work of other investigators is discussed in the light of the present work and the position taken that the total oil deposit is the preferable quantity to measure in the study of the insecticidal action of oils.

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INHERITANCE OF RESISTANCE TO THE HESSIAN FLY IN THE WHEAT CROSSES DAWSON × POSO AND DAWSON × BIG CLUB [1]

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INTRODUCTION

A promising and economical means of controlling the injury to commercial California wheats caused by the hessian fly, *Phytophaga destructor* (Say), lies in breeding varieties resistant to its attack. Such an objective is achieved most easily when the plant breeder uses resistant varieties in which resistance is due to the smallest possible number of factors. The existence of fly-resistant varieties among the common wheats has been demonstrated by many workers. That such resistance is due to a definite number of genetic factors has not yet been shown conclusively. In this preliminary paper data from two crosses are presented which the writers believe indicate the existence of two such genetic factors.

This breeding program was started to develop fly-resistant club wheats for the Montezuma Hills district of Solano County, Calif. It is desirable to grow a club type of wheat in this district, because such wheat does not suffer from shattering from the high winds which prevail there. The two club wheats, Big Club and Poso, which are now grown there are susceptible to the hessian fly. The aim of the breeding program is therefore to add the character of fly resistance to these two varieties and at the same time retain the desirable qualities that they now possess. To attain this end backcrosses will be made to the commercial type and the hybrids thus obtained subjected to rigorous selection under heavy fly infestation.

REVIEW OF LITERATURE

Not a single instance has been found in the literature where the inheritance of resistance to the hessian fly has been definitely put on a factorial basis. The following two statements, however, have a bearing on the problem. Painter, Salmon, and Parker [2] state:

> The most important fact brought out by these data is that resistance is an inherited character which may be combined with other desirable ones and that fly resistance is not closely linked with any observed agronomic character such as awn type and kernel texture.

Later Parker and Painter [3] stated that—

> Studies of crosses between resistant and susceptible wheat varieties have shown that fly resistance is a heritable character, probably governed by multiple factors * * *.

[1] Received for publication Jan. 31, 1936; issued June 1936.

[2] PAINTER, R. H., SALMON, S. C., and PARKER, J. H. RESISTANCE OF VARIETIES OF WINTER WHEAT TO HESSIAN FLY, PHYTOPHAGA DESTRUCTOR (SAY). Kans. Agr. Expt. Sta. Tech. Bull. 27, 58 pp., illus. 1931. See p. 39.

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MATERIAL AND METHODS

The parents used in the crosses under discussion are the susceptible varieties Poso (C. I. no. 8891)[4] and Big Club (C. I. no. 11761) and the resistant variety Dawson (C. I. no. 3342). Pure lines of all parents were used. Poso and Big Club are white-kerneled club varieties having the spring habit type of growth and are well adapted to the Montezuma Hills district. Dawson is a white-kerneled, lax-headed variety having the winter habit type of growth.

The original crosses were made in 1931 at Davis, Calif. The F_1 and F_2 generations were grown at the same place in 1932 and 1933. For the purpose of genetic interpretation the F_2 plants were classified on the basis of their behavior in F_3 rows. The F_3 rows, averaging 75 plants per row, were grown at Birds Landing, in the fly-infested Montezuma Hills district. The seed was sown in December 1933, and the plants were harvested in May and June 1934. The plants therefore made their growth during the winter and early spring and were in receptive condition at the time of infestation, which is normally heavy in that locality. The susceptible and resistant parents were included alternately every eleventh row as checks among the F_3 rows for each cross. A population of F_2 plants of each cross was also grown in 1934.

In the vicinity of Birds Landing the hessian fly usually makes its major flight between the last of February and the early part of April, while the plants are developing from the rosette through the jointing stage. A second flight, from a supplementary brood of flies, generally occurs late in April, or early in May in wet years. Since the natural infestation is usually very high in this district, it has not been necessary to supplement the natural infestation with an artificial one.

The plant was used as the unit in making genetic interpretations. The plants were classified as infested or noninfested depending on whether or not puparia were present. A plant bearing no puparia was considered as resistant.

It will be shown later that a small percentage of plants of the susceptible parents were not infested. These are believed to have escaped infestation, and are not considered as resistant. Packard [5] showed that when progeny of such escaped plants were tested the following year, they did not possess any decidedly resistant qualities. Although he obtained one plant selection which showed a consistently lower percentage of infestation, there are good reasons for believing that this selection was not of the original parent variety but was either a field hybrid or an admixture.

EXPERIMENTAL RESULTS

DETERMINATION OF FACTORIAL BASIS FOR INHERITANCE OF RESISTANCE

The percentages of plants of the parent varieties having hessian fly puparia for the period 1932–34 are given in table 1. The Dawson parent shows a negligible infestation as compared with the other two varieties. The Dawson parent has been tested for a longer period than table 1 indicates, and has always shown a very low infestation. Likewise Big Club has always shown a high infestation over a longer period. The data for the Poso parent do not extend beyond the 3-year period.

[4] Accession number of the Division of Cereal Crops and Diseases, Bureau of Plant Industry.

[5] PACKARD, C. M. THE HESSIAN FLY IN CALIFORNIA. U. S. Dept. Agr. Tech. Bull. 81, 26 pp., illus. 1928.

TABLE 1.—*Infestation of hessian fly puparia in Dawson, Poso, and Big Club wheats, when grown in the field at Birds Landing, Calif., for the years 1932–34*

Variety	1932		1933		1934		Average plants with puparia, 1932–34 [1]
	Plants examined	Plants with puparia	Plants examined	Plants with puparia	Plants examined	Plants with puparia	
	Number	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Percent*
Dawson	100	1.0	50	0.0	760	0.4	0.4
Poso	100	90.0	89	84.3	869	96.9	95.2
Big Club	100	92.0	195	91.8	500	98.4	96.6

[1] Weighted averages.

The F_1 plants from the two crosses were not tested for fly reaction. An F_2 population of 160 plants of Dawson × Poso and 125 plants of Dawson × Big Club was grown subject to fly infestation in 1934. The data for both the present varieties and the crosses are shown in table 2.

TABLE 2.—*Fly-infested plants in the parents and the F_2 generation of the crosses Dawson × Poso and Dawson × Big Club, when grown in the field at Birds Landing, Calif., in 1934*

Parent or cross	Total plants	Fly-infested plants	
	Number	*Number*	*Percent*
Dawson	760	3	0.4
Poso	869	842	96.9
Big Club	500	492	98.4
Dawson × Poso	160	22	13.7
Dawson × Big Club	125	23	18.4

As indicated previously, the genetic classification of the F_2 plants is based on their behavior in F_3 rows. There were 185 F_3 rows grown of each cross. The fly infestation was uniform throughout the nursery, as indicated by the parent rows which were included alternately every eleventh row as checks. The percentages of fly infestation for the alternate parent checks were as follows: In the Dawson × Poso nursery, 99, 1, 97, 0, 100, 0, 94, 0, 95, 0, 97, 0, 99, 0, 94, 1, 97, 1, 98; and in the Dawson × Big Club nursery, 100, 0, 100, 0, 96, 0, 98, 0, 98, 0, 98, 0, 96, 0, 98, 0, 100, 0, 100. The low percentages are for Dawson. The data for the infestation of the F_3 rows of both crosses are summarized in 5-percent classes in table 3, and are shown graphically in figure 1.

On the assumption of an arbitrary minimum at 77.5 percent infestation, this point was used in dividing the rows into two groups, according to whether more or less than 77.5 percent of the plants were infested. Since only 10 rows were grown of each susceptible parent, the range distribution for such rows is not well established. Therefore, if only those hybrid rows are considered susceptible which fall within the range of the susceptible parent, some errors of classification might result. Again, the range of susceptibility of the rows of susceptible parents is based on the relation of the two postulated

susceptible genes to a constant reaction system of other genes. Since this is not the case in rows of hybrids, the range may be extended so that a minimum somewhat beyond the range of the susceptible parent may be justified.

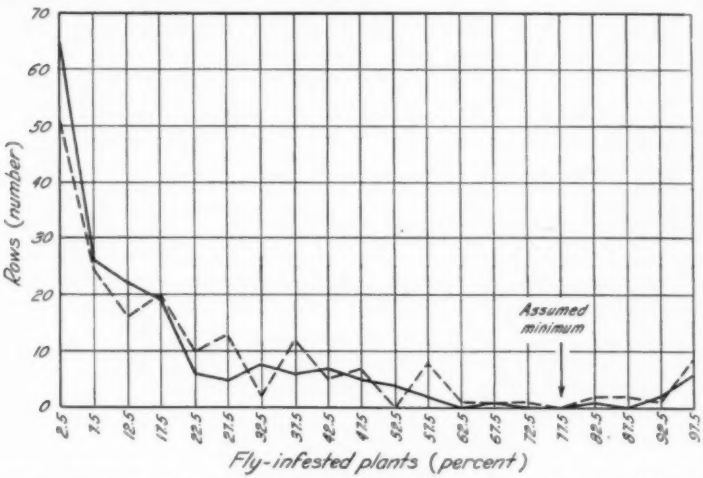

FIGURE 1.—Distribution of F_3 rows of the crosses Dawson × Poso (solid line) and Dawson × Big Club (broken line) in 5-percent classes of infestation with hessian-fly puparia.

TABLE 3.—*Distribution of the parent and F_3 rows of the wheat crosses Dawson × Poso and Dawson × Big Club in 5-percent classes of hessian fly infestation, when grown in the field at Birds Landing, Calif., in 1934*

Parent or cross	Rows in indicated classes of fly infestation [1]										
	2.5 percent		7.5 percent	12.5 percent	17.5 percent	22.5 percent	27.5 percent	32.5 percent	37.5 percent	42.5 percent	47.5 percent
	0 percent	0+ to 5 percent									
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
Dawson	15	3									
Poso											
Big Club											
Dawson × Poso	19	46	26	22	19	6	5	8	6	7	5
Dawson × Big Club	18	33	24	16	20	10	13	2	12	5	7

Parent or cross	Rows in indicated classes of fly infestation [1]										Total rows
	52.5 percent	57.5 percent	62.5 percent	67.5 percent	72.5 percent	77.5 percent	82.5 percent	87.5 percent	92.5 percent	97.5 percent	
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
Dawson											18
Poso									2	8	10
Big Club										10	10
Dawson × Poso	4	2	0	1	0	0	1	0	2	6	185
Dawson × Big Club	0	8	1	1	1	0	2	2	1	9	185

[1] Percentages given are class centers.

The 185 F_3 rows (or F_2 plants) of each of the two crosses then group themselves into the ratios 176:9 and 171:14 for the Dawson × Poso and Dawson × Big Club crosses, respectively. On the basis of a two-factor 15:1 theoretical genetic ratio, the numbers to be expected are 173.4:11.6. The actual and theoretical ratios for both crosses agree within the limits of the experimental error. Under the conditions of these experiments the resistance of Dawson is therefore interpreted as being controlled by two genetic factors.

The interpretation of the data applies only to the conditions under which the experiments were made, and no prediction can be made as to the behavior of the parent or hybrid material when grown under other conditions. The data furnish no idea as to the fundamental nature of resistance. The answer to that problem might be of some help in the breeding program, but it is not necessary for such a program.

ONTOGENY OF THE FLY ON SUSCEPTIBLE AND RESISTANT PLANTS

The normal ontogeny of the hessian fly on susceptible plants may be briefly described as follows: The adult flies emerge from the puparia and mate during February and March, and the females lay their eggs on the leaves of the growing crop. In a few days the red eggs hatch, and the salmon-colored young larvae crawl down between the culm and leaf sheath and begin to feed. After feeding for 2 to 3 weeks, the larvae, then glistening white, form puparia and enter a quiescent stage, or what is commonly called a "flaxseed." During the long, dry summer and fall, these "flaxseeds" remain unchanged in the same stubble and straw that the young larvae had occupied while sucking their food from the growing stems. Late in the winter or early in the spring of the following year the larvae pupate, and in 2 or 3 weeks the adults emerge to repeat the life cycle.

On resistant plants the life cycle is not completed. The life of the insect appears to be normal until the newly hatched red larvae reach the feeding position between the leaf sheath and culm. Shortly after this the larvae die. It is this fact that points to the possibility of producing commercial wheats that will be free from fly attack.

SUMMARY

An attempt is being made in California to breed varieties of wheat that will possess resistance to infestation by the hessian fly. The existence of resistant varieties among common wheats has been demonstrated by many workers, but the number of genetic factors involved in such resistance has not previously been conclusively shown.

Selections from the variety Dawson are shown to be highly resistant to fly attack, and the varieties Poso and Big Club are very susceptible under California conditions. The crosses Dawson × Poso and Dawson × Big Club show inheritance of resistance in the F_2 generation when classified on their behavior in F_3 rows, in a ratio closely approximating the theoretical 15:1 ratio occurring when two factors are involved.

The ontogeny of the hessian fly in relation to both resistant and susceptible varieties is discussed briefly.

Under the conditions of the experiments as described in the text, the data for the two crosses studied indicate that resistance to the hessian fly in the Dawson variety is heritable and that it is controlled by two genetic factors.

QUANTITATIVE DEMONSTRATION OF THE PRESENCE OF SPORES OF BACILLUS LARVAE IN HONEY CONTAMINATED BY CONTACT WITH AMERICAN FOULBROOD[1]

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INTRODUCTION

In a previous paper[3] the writer showed that it is possible to demonstrate the presence of spores of *Bacillus larvae*, the cause of American foulbrood, in samples of commercial honey that have had contact with American foulbrood in the course of their production or preparation for the market. Since this work was reported, 25 additional samples, making a total of 212 samples of commercial honey, obtained on the open market from 28 States and 2 Territories have been examined by the same method, and spores of *B. larvae* have been found in 17, or 8 percent, of these samples.[4] In most cases the spores were present in relatively small numbers.

The method of examination used in the work thus far reported gave only a qualitative indication of the number of spores present, the observations being recorded as showing "the presence of a sufficient number of spores resembling spores of *B. larvae* to be designated as positive."[5] This amounted to from one or two definite spores to a very few spores seen in numerous microscopic fields of each stained sediment examined. The primary object was to demonstrate only their presence or absence. It was assumed that in most cases the number of spores found was considerably smaller than would be found in honey containing numbers comparable with the observed minimum infective dose of 50,000,000 per liter.

The only way of demonstrating the accuracy of this assumption has been to feed such "positive" samples of commercial honey to healthy colonies of bees. This was done with 15 of the 16 samples in which spores were demonstrated, and only 1 sample, or 6.7 percent, was found to contain sufficient infection to produce the disease in a healthy colony. These investigations indicate that the requirement of certification of honey, as has been proposed and even placed in operation in certain States, is not a justifiable measure in the control of American foulbrood under the present conditions of inspection and control of disease in this country.

To permit a more accurate, quantitative study of the infectivity of honey that has been in contact with American foulbrood, on the

[1] Received for publication Jan. 27, 1936; issued June 1936. This investigation was carried on at the Intermountain States laboratory of the Division of Bee Culture, which is maintained cooperatively by the University of Wyoming and the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

[2] Acknowledgments are due to F. R. Hall, associate professor of commerce, University of Wyoming, for advice and assistance in the statistical analysis of the data.

[3] STURTEVANT, A. P. RELATION OF COMMERCIAL HONEY TO THE SPREAD OF AMERICAN FOULBROOD. Jour. Agr. Research 45: 257–285, illus. 1932.

[4] STURTEVANT, A. P. HONEY OF THE INTERMOUNTAIN REGION. Gleanings Bee Cult. 63: 463–468, illus. 1935.

[5] STURTEVANT, A. P. See footnote 3.

basis of its spore content—that is, a detailed study of the distribution of spores of *B. larvae* in the honey from infected hives or apiaries, or in commercial honey obtained on the open market, or of the effect of mixing infected honey with disease-free honey in the course of production or blending and preparation for the market—a more detailed investigation has been made of the spore content of honey containing approximately known numbers of spores. This has been accomplished by an improved and more accurate method of determining the number of spores in such honey, and the accuracy of the results and method has been demonstrated by means of a statistical analysis of the data obtained.

METHOD OF OBTAINING THE DATA

PREPARATION OF SAMPLES OF HONEY

A series of samples of honey containing approximately known numbers of spores per cubic centimeter were prepared in the manner described previously,[6] by adding to 100-cc quantities of spore-free honey the necessary quantities of various dilutions of a stock suspension of spores of *Bacillus larvae* containing approximately 5,000,000,000 spores per cubic centimeter. Five samples of honey were prepared in this way containing approximately 1,000,000, 800,000, 500,000, 300,000, and 50,000 spores per cubic centimeter, respectively. These samples, each considered as a unit and not as a dilution of the 1,000,000-spore sample, were heated in a water bath to 120°–130° F., and then thoroughly mixed with a mechanical stirrer for 5 minutes. Duplicate 5-cc quantities of each sample were then placed in 50-cc conical centrifuge tubes, and 45 cc of distilled water of approximately the same temperature was added. When the honey and water were completely mixed, the samples were centrifuged at 2,000 revolutions per minute for 45 minutes. All but about 1 cc of the supernatant honey-water solution of each sample was then removed by means of a pipette and suction. Again approximately 45 cc of distilled water was added, and after thorough mixing the suspensions were centrifuged for 30 minutes longer. The removal of the supernatant solution was repeated until all but approximately 0.1 cc[7] of the water had been removed from each centrifuge tube, and each sample of sediment was completely suspended in this remaining quantity of water by blowing gently through a capillary pipette dipped into the water. Duplicate 0.01-cc quantities of each suspension were then transferred with the capillary pipette (calibrated to deliver 0.01 cc) to microscope cover glasses. Circular cover glasses, size 12, no. 1 thickness, having an area of 1.13 cm^2, proved satisfactory for this purpose. A small (2 to 3 mm) loopful of carbolfuchsin stain was added to the drop of suspension on the cover glass and thoroughly mixed with it. This stained liquid was then spread uniformly over a 1-cm^2 area of the cover glass, a narrow ring at the outside edge being left uncovered. The smears were allowed to dry in the air and were then mounted on microscope slides either with water or, preferably, with Canada balsam, for examination under the microscope. These stained smears were not washed in water, as this might have caused some spores to be lost.

[6] STURTEVANT, A. P. See footnote 3.
[7] A mark was placed on the outside of the conical centrifuge tubes to indicate the 0.1-cc volume.

The foregoing process gives a concentration of spores in the sediment from the 5-cc samples of honey suspended in 0.1 cc of water, or one-fiftieth the original volume.

METHOD OF COUNTING SPORES

A method similar to that of Breed and Brew [8] for counting bacteria in milk was used for counting the spores of *Bacillus larvae* in these stained smears. This method is similar to that described in a previous paper [9] and is represented by the formula

$$\text{Number of spores per cubic centimeter} = \frac{KNX \times 100 \times D}{N}$$

where K is the factor for the number of circular fields per 1-cm^2 area, N is the number of circular fields counted, X is the actual mean number of spores per field, 100 is the factor that gives the number of spores per cubic centimeter from 0.01 cc of the suspension, and D is the dilution.

TABLE 1.—*Spore counts in stained smears of the sediments resulting from the centrifuging of duplicate 5-cc portions of five samples of honey containing known numbers of spores of Bacillus larvae*

Field no.	Spore counts in samples [1] containing the indicated number of spores per cubic centimeter									
	50,000		300,000		500,000		800,000		1,000,000	
	A	B	A	B	A	B	A	B	A	B
1	2	1	7	8	14	15	19	21	24	21
2	2	2	8	9	12	12	18	20	24	29
3	1	1	8	10	12	13	24	18	26	38
4	0	2	7	5	10	15	22	18	23	24
5	2	0	9	6	10	11	20	20	30	34
6	1	3	9	7	12	13	18	17	23	29
7	0	1	8	7	15	14	21	18	26	24
8	2	1	7	8	12	13	27	17	29	21
9	1	0	8	6	16	16	19	21	19	26
10	0	2	9	12	17	13	21	20	30	31
11	1	1	7	11	14	12	16	19	25	36
12	1	3	10	6	16	12	21	20	28	26
13	1	3	9	5	13	10	22	22	27	33
14	2	1	7	9	18	14	25	24	25	22
15	1	0	8	10	12	13	21	25	29	26
16	2	1	8	5	10	11	20	25	24	26
17	1	0	6	9	11	16	18	28	35	23
18	2	0	5	6	13	14	26	23	24	28
19	0	2	7	10	17	16	24	23	25	34
20	0	1	9	5	13	18	26	28	27	30
21	2	1	10	15	13	15	16	23	25	32
22	2	3	11	10	8	16	19	18	26	25
23	1	1	7	8	18	10	20	16	27	25
24	3	1	6	5	15	12	18	22	28	34
25	1	1	7	9	12	15	21	21	29	28
26	0	1	6	7	15	10	21	20	27	22
27	1	1	5	10	10	14	26	18	34	23
28	2	1	7	8	12	15	22	26	22	27
29	2	2	8	10	11	11	22	28	29	28
30	2	2	10	8	12	11	25	22	21	30
Total	38	39	233	244	393	400	638	641	791	835
Total for 60 fields	77		477		793		1,279		1,626	
Mean number of spores per field	1.2833		7.9500		13.2167		21.3167		27.1000	

[1] A and B represent duplicate portions of the samples.

[8] BREED, R. S., and BREW, J. D. COUNTING BACTERIA BY MEANS OF THE MICROSCOPE. N. Y. State Agr. Expt. Sta. Tech. Bull. 49, 31 pp., illus. 1916.

[9] STURTEVANT, A. P. See footnote 3.

An ocular micrometer disk, such as is used for counting bacteria in milk, was used in counting spores in the fields of the stained smears. The area of the circle etched on this disk was found to be 0.00006082 cm^2 when used in a binocular microscope with 15 × paired eyepieces and a 1.8-mm oil-immersion objective. Therefore, the factor K became 16,441.96.

The spores in 30 fields from each of the duplicate smears were counted, making a total of 60 fields (N) for each honey-spore sample. The fields were counted at random from various parts of the smear. From these counts the actual mean number of spores per field recovered in 60 fields for each honey-spore sample was determined (table 1).

Substituting the values for K and N and 0.02 (1/50) for D, the spore dilution in the foregoing formula gives

Number of spores per cubic centimeter

$$=\frac{16,442\times 60X\times 100\times 0.02}{60}=32,884X$$

COMPUTATION OF THEORETICAL MEAN NUMBER OF SPORES PER FIELD

The theoretical mean numbers of spores per field that should be recovered from each of five honey-spore samples used, under ideal conditions where there is no loss of spores during the process, were calculated by the foregoing formula, which for this purpose may be stated as follows:

$$X=\frac{\text{Number of spores per cubic centimeter}}{32,884}$$

X now designates the theoretical mean number of spores per field. In table 2 these values are given in comparison with the corresponding actual mean number of spores per field for each honey-spore sample.

TABLE 2.—*Relation between the actual and the theoretical mean numbers of spores of Bacillus larvae per field recovered from five samples of honey containing known numbers of spores per cubic centimeter*

Spores per cubic centimeter in sample (number)	Mean spores per field			Ratio of actual mean to theoretical mean
	Theoretical	Actual	Standard deviation	
	Number	*Number*	*Number*	*Percent*
1,000,000	30.4100	27.1000±0.3554	4.0812	89.12
800,000	24.3279	21.3167± .2751	3.1596	87.62
500,000	15.2050	13.2167± .2011	2.3100	86.92
300,000	9.1230	7.9500± .1708	1.9615	87.14
50,000	1.5205	1.2833± .0747	.8582	84.40

RESULTS OBTAINED BY USE OF THE METHOD

By the method used, the actual mean number of spores per field obtained by counting 60 fields from each honey-spore sample differed from the calculated theoretical mean number of spores per field by 10.88 percent for the honey containing 1,000,000 spores per cubic centimeter to 15.60 percent for the honey containing 50,000 spores per

cubic centimeter (table 2). This difference, which is relatively constant for each sample, may be due to the fact that some spores are lost during the centrifuging, but more probably to the fact that a certain proportion of the spores in each smear are covered up and not seen in the masses of stained debris always present even in honey of the highest quality.

DETERMINATION OF ACCURACY OF THE METHOD

STATISTICAL ANALYSIS OF THE DATA

Since the data obtained for the actual mean number of spores per field (table 1) for each honey-spore sample, if plotted against the data calculated for the theoretical mean number of spores per field (table 2), give practically a straight line having a trend similar to that of a line plotted for the theoretical data alone, the relation between the theoretical means and the actual means, for the five honey-spore samples used, was determined by the customary statistical methods.

The standard deviation and the probable error for the actual mean number of spores per field were determined from frequency tables prepared from the original data (table 1) for each honey-spore sample used [10] (table 2). The actual means were derived from large samples (60 fields each), and the calculated probable errors and standard deviations were shown statistically to be small.

The coefficient of correlation [11] between the values for the actual mean number and those for the theoretical mean number of spores per field for each sample as given in table 2 was found to be 0.9999 ± 0.0001.

The relation between the actual mean number of spores per field recovered from each honey-spore sample and the corresponding most probable values estimated from the theoretical mean number of spores per field for each sample was determined by use of the regression equation for the actual mean number of spores. This was found to be $\overline{Y}=0.8905X-0.1791$. Substituting the various values of the theoretical mean number of spores per field (table 2) for X in this equation gave the most probable estimated values for the actual mean number of spores per field ($\overline{Y}$) that should have been recovered from each sample (table 3). These most probable estimated values were found to be in excellent agreement with the actual values obtained.

TABLE 3.—*Theoretical and actual mean numbers of spores per field and the most probable estimated theoretical and actual mean numbers of spores per field*

Number of spores per cubic centimeter in sample	Mean number of spores per field			
	Theoretical	Estimated theoretical	Actual	Estimated actual
1,000,000	30.4100	30.6313	27.1000	26.9010
800,000	24.3280	24.1378	21.3167	21.4850
500,000	15.2050	15.0431	13.2167	13.3610
300,000	9.1230	9.1297	7.9500	7.9449
50,000	1.5205	1.6443	1.2833	1.1749

[10] CHADDOCK, R. E. PRINCIPLES AND METHODS OF STATISTICS. pp. 160–164, 240–241. Boston, New York [etc.]. 1925.

[11] CROXTON, F. E., and COWDEN, D. J. PRACTICAL BUSINESS STATISTICS. p. 416. New York. 1934.

The purpose of this investigation, however, was to develop an equation with which, if the actual mean number of spores per field is obtained with sufficient accuracy, the theoretical number of spores per field may be estimated, thereby giving the data necessary for estimating the number of spores per cubic centimeter in an unknown sample of honey. The regression equation or the theoretical mean number of spores per field can be used for this purpose, and was found to be $\overline{X}=1.1228Y+0.2034$. Substituting for Y in this equation, the various values of the actual mean number of spores per field, as obtained in table 1, gave the most probable estimated values for the theoretical mean number of spores per field that should be obtained from the actual counts for each honey-spore sample (table 3). By this method of estimation these values were found to agree closely with the original calculated values for the theoretical mean number of spores per field for each honey-spore sample (table 2).

DETERMINATION OF PERMISSIBLE LIMITS OF ERROR

The analysis of the data so far indicates the accuracy of the method outlined above for determining the most probable actual mean spore count per field from the mean of 60 fields counted. Variations in the counts may occur in individual samples, however, owing to the failure to recover all the spores, as stated previously.

The permissible limits of error in the statistical analysis of such cases are customarily determined by use of the standard error of estimate. This, for the most probable estimated actual means derived from the theoretical means, was found to be small, ± 0.1298 spore, and indicates the closeness with which new estimated values may be expected to approximate the true but unknown values. Since two of the five actual means fall within ± 0.1298 spore of the estimated actual means while the other three are only from 0.11 to 0.26 percent outside this zone, within which approximately two-thirds of the observations may be expected to fall in relation to the most probable values, a sufficient accuracy for the method is indicated.

The standard error of estimate for the most probable theoretical means derived from the actual means (which were found to agree closely with the estimated actual means) was found to be ± 0.1458 spore. As is to be expected in this case, again two of the original theoretical means fall within the zone of ± 0.1458 spore while the other three are only from 0.11 to 0.25 percent outside this zone. However, since ± 3 times the standard error of estimate, which should include 99.7 percent of all observations, is used customarily in delineating the largest error to which statistical analyses of this type are subject, it is found that all the theoretical means fall well within this zone, or within ± 0.4374 spore. This indicates the probable accuracy of estimating the number of spores per cubic centimeter in an unknown sample by calculating the most probable theoretical number of spores per field from the actual mean number counted.

PRACTICAL APPLICATION OF THE METHOD

In a previous paper [12] it was shown that during observations covering 5 years no cases of American foulbrood developed in 19 colonies of bees fed less than approximately 50,000,000 spores of *Bacillus*

[12] STURTEVANT, A. P. See table 1 of reference in footnote 3.

larvae in 1 liter of sugar sirup, or less than 50,000 spores per cubic centimeter. Of 11 colonies fed 50,000 spores per cubic centimeter, 2 developed disease and 9 remained healthy; of 6 colonies fed 75,000 per cubic centimeter, 3 developed positive disease and 1 probable disease, and 2 remained healthy; of 6 colonies fed 100,000 per cubic centimeter, 2 were positive, 1 probable, and 3 remained healthy; of 4 colonies fed 200,000 spores per cubic centimeter, 3 were positive and 1 probable. Thus it was assumed that 50,000 spores per cubic centimeter of sirup could be considered the critical number or minimum infectious dose of spores that will produce disease, when 1 liter is used as the unit volume to be fed.

Since the foregoing analysis of the data indicates, by the method of estimating used, that the actual mean number of spores per field falls well within the limits of permissible error for the estimated actual means (± 3 times the standard error of estimate), the most probable value for such a mean for use in determining the number of spores per cubic centimeter of an unknown sample is the actual mean number of spores per field determined by counting 30 fields each from stained smears from two centrifuged sediments of this sample. If the formula $X=1.1228Y+0.2034$ is used to estimate X, the most probable theoretical number of spores that should have been recovered, when Y represents the actual mean number of spores per field, and if this value is then multiplied by 32,884, the most probable number of spores per cubic centimeter in the unknown sample can be calculated. Applying the limits of error for X, ± 3 times the standard error of estimate, or ± 0.4374 spore, and carrying it through into the second formula will give the possible range in which the number of spores per cubic centimeter might fall within the precision of the method.

Further work is in progress to determine whether the same accuracy will be obtained by counting a smaller number of fields to obtain the mean number of spores per field from a larger number of smears from sediments.

Since in the experimental work the samples of known spore content contained approximately round numbers of spores—multiples of 50,000—it probably would be sufficiently accurate to designate the number of spores as the nearest multiple of 50,000 to the actual figures derived from the formulas. When using the limits of error 0 ± 0.4374 spore per field, for the estimated mean number of spores per field, it will be found that for numbers below 100,000 there will be some overlapping between 10,000-spore increments, and the value will have to be expressed approximately (for example, the honey contains between 40,000 and 60,000 spores per cubic centimeter); nevertheless the honey can still be designated either as dangerous or as not dangerous.

SUMMARY

Previous work on the qualitative demonstration of the presence or absence of spores of *Bacillus larvae* in honey that has been in contact with American foulbrood has been followed by the development of a quantitative method for determining the approximate number of spores per cubic centimeter in such honey. The method is represented by the formula

$$\text{Number of spores per cubic centimeter}=\frac{KNX\times 100\times D}{N}$$

where K is the factor for the number of circular fields per 1-cm^2 area, N is the number of circular fields counted, X is the actual mean number of spores per field, 100 is the factor that gives the number of spores per cubic centimeter from 0.01 cc of the suspension, and D is the dilution. The mean number of spores of *Bacillus larvae* per field counted in 60 fields of stained smears made from the sediments obtained by centrifuging 5-cc quantities of honey containing approximately known numbers of spores have been determined by this method.

The mean actual spore count per field was determined for a series of samples of honey prepared to contain approximately 1,000,000, 800,000, 500,000, 300,000, and 50,000 spores per cubic centimeter. The mean theoretical spore count per field that should have been recovered was determined by use of the formula

$$X=\frac{\text{Number of spores per cubic centimeter}}{32{,}884}$$

The actual mean numbers of spores per field were similar in trend to the calculated theoretical means but were from 10.88 to 15.60 percent smaller. A statistical analysis of the data to determine the accuracy of the method showed that the calculated probable errors and standard deviations were small. The coefficient of correlation between the actual and the theoretical mean number of spores per field for each sample was found to be 0.9999 ± 0.0001.

The relation between the actual mean number of spores per field (Y) and the corresponding most probable values that should have been recovered, estimated from the theoretical mean number of spores per field (X), was determined by means of the regression equation $\overline{Y}=0.8905X-0.1791$. These most probable estimated values were found to be in excellent agreement with the actual values obtained, well within the customary limits of ± 3 times the standard error of estimate, which was found to be ± 0.1298 spore.

The most probable theoretical mean number of spores per field (X) was estimated by means of the regression equation $\overline{X}=1.1228Y+0.2034$. These values were found to be in excellent agreement with the original calculated values for the theoretical mean, well within ± 3 times the standard error of estimate, ± 0.1458 spore.

The statistical analysis of the data therefore indicates that the method used is sufficiently accurate for determining the spore content of unknown samples of honey. For this purpose the following formulas are used:

$$\overline{X}=1.1228Y+0.2034 \pm 0.4374$$

where Y = the actual mean number of spores per field counted from 60 fields, and

$$\text{Number of spores per cubic centimeter}=32{,}884X.$$

THE TOXIC EFFECTS OF NAPHTHALENE ON BRUCHUS OBTECTUS AND TENEBRIO MOLITOR IN VARIOUS STAGES OF DEVELOPMENT[1]

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INTRODUCTION

Although naphthalene has been in use as an insecticide for about 50 years, knowledge of its toxic effects is based mainly on investigations conducted under uncontrolled conditions. Very little is known of the physiological effects of naphthalene on insects. In late years naphthalene has been found useful against an increasing variety of insect pests, but it is probably most effective in killing or repelling insects found in houses, greenhouses, stored products, and in the soil.

The period of exposure necessary to kill the immature forms of the Japanese beetle in a saturated atmosphere of naphthalene depends on the temperature (Fleming and Baker (*3*)).[2] It ranges from 12 hours at 80° F. to 120 hours at 50°. The relative humidity of the atmosphere also influences insecticidal action on the larvae, mortality increasing with an increase in relative humidity.

Herrick and Griswold (*5*) found that naphthalene inhibited the development of the eggs of clothes moths, no eggs hatching after an exposure of 14 days in an enclosed space at room temperatures. Hartzell and Wilcoxin (*4*) observed that naphthalene was toxic to the eggs of the red spider mite. Read (*8*) showed by laboratory experiments that at least 8 hours' exposure of red spider mite eggs to a saturated atmosphere was required to prevent hatching. A study of the comparative resistance of the larva, protonymph, deutonymph, and adult female to naphthalene vapor showed that there was a slight increase in resistance as the stages advanced from larva to adult. Fleming and Baker (*3*) found the resistance of immature stages of the Japanese beetle to increase in the following order: (1) larvae, (2) eggs, (3) pupae. It was observed by Mercier (*7*) that, when the pupae of the fly *Calliphora erythrocephala* Meig. had been exposed to naphthalene vapor, some of the first-generation descendants of the treated flies showed malformations, but in the second generation these malformations did not appear. Shull, Riley, and Richardson (*10*) found that naphthalene produced no apparent effects on the coagulation of the blood nor on the appearance of the blood cells in the oriental cockroach (*Blatta orientalis* L.): Fleming and Baker (*3*) observed that eggs and larvae of the Japanese beetle exposed to naphthalene vapor became reddish in color, the intensity of the color depending on the period of exposure. The vapor appeared to have a paralyzing effect on the larvae of the Japanese beetle. Toscano

[1] Received for publication Nov. 25, 1935; issued June 1936. Based on a thesis presented by the senior author to the faculty of Cornell University in partial fulfillment of the requirements for the degree of doctor of philosophy.

[2] Reference is made by number (italic) to Literature Cited, p. 712.

Rico (*11*) also observed the paralyzing effect of naphthalene vapor on the parasitic roundworm (*Ascaris lumbricoides* L.). This paralysis was followed by death.

In view of the more extended use which might be made of naphthalene in the future, a clearer knowledge of the toxic effects of this material would be of much value to entomologists. For this reason investigations on the toxicity of naphthalene to various stages of the bean weevil and yellow mealworm have been conducted under controlled conditions, and the findings are here presented.

METHODS

The apparatus used in the experiments was a modification of that used by Lehman (*6*). Humidity was controlled by passing air through saturated solutions of sodium chloride to give the air stream a relative humidity of approximately 70 percent. The apparatus was placed in a chamber the temperature of which was held constant at 25° C. by the use of a bimetallic thermoregulator with a resistance unit as a source of heat. In all experiments air was passed through this apparatus at the rate of 14 liters per hour. To determine whether saturation of the air was obtained, U-tubes containing naphthalene were weighed and placed in the system and then reweighed after the air had passed through them for a definite length of time. The loss in weight of the U-tubes was the amount of naphthalene taken up by the air. The amount volatilizing in a definite volume of air checked closely with the calculations for the amounts of naphthalene in a saturated atmosphere published by Roark and Nelson (*9*).

Before the beginning of each experiment air was passed over the naphthalene for about one-half hour. The insects were then placed in the flasks and allowed to remain there the desired length of time in contact with a constant stream of air saturated with naphthalene. Flasks containing control insects were similarly treated except that they were not exposed to naphthalene vapor. At the conclusion of each experiment the insects were removed and placed in a rearing chamber at a constant temperature and humidity. All insects in their various stages before and after treatment were kept in the constant-temperature chamber at 25° C. with a relative humidity of approximately 60 percent.

Bean weevils (*Bruchus obtectus* Say) were reared on red kidney beans at a temperature of 25° C. and relative humidity of 60 percent. The cultures were started at weekly intervals so that a new batch of adults might emerge every week.

Larvae of the yellow mealworm (*Tenebrio molitor* L.) were reared on a dry, complete dog food in the form of a meal, while the adults were fed once a week on dog biscuits, canned dog food, and banana skins. The cultures were kept at a constant temperature of 27.5° C. and relative humidity of 70 percent.

EFFECTS OF NAPHTHALENE ON EGGS OF THE BEAN WEEVIL AND YELLOW MEALWORM

A series of experiments was undertaken to determine the toxicity of naphthalene vapor to the eggs of insects. An attempt was made to determine whether the toxicity varied with the age of the eggs and whether any general physiological effects were produced.

Bean weevil and yellow mealworm eggs, none of which were over 1 day old, were exposed to naphthalene vapor for varying periods of time. The percentage of eggs hatching in the checks was very constant, averaging 90 percent or more (table 1). Naphthalene was toxic to the eggs, the number which failed to hatch varying directly with the period of exposure to the vapor. The large mealworm eggs were affected much more readily when not more than 1 day old than were the small eggs of the bean weevil of the same age. Approximately the same lethal effects were obtained with eggs of the mealworm with only one-half the length of exposure given the bean-weevil eggs.

TABLE 1.—*Toxicity of naphthalene* [1] *to eggs of the bean weevil and the yellow mealworm at different time exposures*

Exposure	Trials	Check		Treated	
		Total eggs	Hatched	Total eggs	Hatched
Hours	*Number*	*Number*	*Percent*	*Number*	*Percent*
BEAN-WEEVIL EGGS 0 TO 1 DAY OLD					
1	3	300	96	1,000	87
2	3	400	91	1,000	80
3	9	1,000	93	2,300	45
4	4	600	91	1,200	14
5	4	500	92	1,300	6
6	4	400	95	1,200	.4
MEALWORM EGGS 0 TO 1 DAY OLD					
½	2	200	90	600	85
1	3	300	89	700	72
1½	3	300	89	800	48
2	3	300	91	1,000	25
2½	3	300	85	900	8
3	5	455	91	735	1

[1] Naphthalene-saturated air stream at 25° C.; relative humidity, 70 percent.

To determine the relation between the stage of development of an insect egg and its susceptibility to the vapor of naphthalene, eggs of both the bean weevil and the mealworm were exposed for 3-hour periods at different ages (table 2). A minimum of 500 eggs was used at each stage of development and several replicates and checks were run with each experiment. The percentage of bean-weevil eggs killed increased with age until the eggs were 4 to 5 days old, when the peak was reached. From that time on toxicity decreased and when the eggs were ready to hatch (6 to 7 days old) very few were killed. The reaction of mealworm eggs to naphthalene vapor was almost the reverse of that of the bean-weevil eggs. The gas was most lethal to eggs 1 day old, with a sudden decrease in toxicity thereafter and a slight increase just before hatching (7 to 8 days old). Newly emerged larvae showed greater resistance than did the eggs to the vapors of naphthalene. These results indicate that the age or stage of development of an insect egg influences its susceptibility to naphthalene.

TABLE 2.—*Age and percentage daily loss in weight of bean weevil and yellow mealworm eggs in relation to the toxicity of naphthalene vapors*

Age of eggs	Eggs dead after 3-hour exposure		Daily loss in weight of untreated normal eggs		Age of eggs	Eggs dead after 3-hour exposure		Daily loss in weight of untreated normal eggs	
	Bean weevil	Mealworm	Bean weevil	Mealworm		Bean weevil	Mealworm	Bean weevil	Mealworm
Days	*Percent*	*Percent*	*Percent*	*Percent*	*Days*	*Percent*	*Percent*	*Percent*	*Percent*
0 to 1	55	99	2.72	7.83	5 to 6	68	11	3.48	1.38
1 to 2	70	83	2.71	2.08	6 to 7	22	15	----------	1.68
2 to 3	71	38	2.77	1.51	7 to 8	----------	23	----------	2.04
3 to 4	81	12	3.67	1.18	8 to 9[1]	----------	0	----------	1.68
4 to 5	90	10	4.56	1.18	9 to 10[1]	----------	----------	----------	.96

[1] Larvae.

Cotton (*2*) has shown that the effects of fumigants on insects can be correlated with their respiratory metabolism. During the course of their development the insect eggs which were used in these experiments continuously lost weight, presumably through the loss of water and carbon dioxide. Since both water and carbon dioxide are the end products of respiratory metabolism it is believed that the percentage daily loss in weight of the eggs can be used as a relative measure of the rate of respiratory metabolism.

Normal bean weevil and mealworm eggs were cleaned and weighed on a chemical balance and then placed in closed containers over a saturated solution of ammonium nitrate (60 percent relative humidity) at a temperature of 25° C. The eggs were weighed at 24-hour intervals until they hatched, and the percentage of daily loss in weight obtained (table 2). The data indicate that there is a definite relation between weight loss of the eggs (respiratory metabolism) and the lethal effects of naphthalene. A greater weight loss (respiratory metabolism) was associated with increased mortality of the eggs.

Naphthalene vapor in sublethal dosages prolonged the developmental period of bean weevil eggs. The mean number of days required to complete incubation for bean weevil eggs of different ages which survived exposure for 3 hours to a naphthalene-saturated atmosphere is shown in table 3.

TABLE 3.—*Mean number of days required to complete incubation for bean weevil eggs of different ages which survived 3-hour exposure to a naphthalene-saturated atmosphere*

Age of eggs when treated	Incubation period		Age of eggs when treated	Incubation period	
	Untreated	Treated		Untreated	Treated
Days	*Days*	*Days*	*Days*	*Days*	*Days*
1	8.1	9.4	5	7.7	9.1
2	8.0	9.2	6	7.8	8.8
3	7.8	9.1	7	8.0	8.4
4	7.8	9.6			

Exposure in early stages of development delayed hatching of those eggs that survived for more than a day. The greatest delay occurred

when eggs were 3 to 4 days old at about which time naphthalene was most toxic (table 2). When the eggs were ready to hatch (6 to 7 days old) the vapor delayed hatching very little. Those larvae that survived 3-hour exposure to naphthalene were apparently uninjured. The incubation period of the eggs of the mealworm was not significantly affected by the naphthalene treatment. However, the treated eggs, previously creamy white in color, assumed a pink or reddish-brown shade, this color being more prominent in the eggs which had been exposed for the longest period. An examination of the newly emerged larvae from such eggs indicated that this discoloration occurred in the large mid-intestine. No discoloration was observed in other parts of the body as Fleming and Baker (*3*) had found in larvae of the Japanese beetle. The larvae appeared normal in all other respects and the colored matter disappeared after a few days.

EFFECTS OF NAPHTHALENE ON ADVANCED STAGES OF THE YELLOW MEALWORM AND ADULTS OF OTHER SPECIES

Previous data (table 2) showed that after the mealworm eggs had hatched, there was a decided increase in the resistance of the larvae to the lethal effects of naphthalene vapor. Since considerable variation in size was noted among larvae of the same age in the stock cultures, treatments were made with different sized larvae of the same age to determine whether the toxicity of naphthalene vapor was related to the size of the larvae. Larvae of different ages but of the same size were also treated to see whether the age of the larvae affected their susceptibility to naphthalene vapor. After treatment larvae were placed in the constant temperature and humidity cabinet for 48 hours, when the numbers of living and dead insects were recorded. The data (table 4) show that both weight and age influenced larval susceptibility to naphthalene. Sublethal exposures to naphthalene vapor of the newly emerged larvae of the mealworm did not affect subsequent growth as measured by gain in weight.

TABLE 4.—*Comparative toxicity of naphthalene to various stages of the yellow mealworm with special reference to size and age of larvae*

16-HOUR EXPOSURES

Stage	Age	Treated	Weight (average)	Weight loss (24 hours)	Dead
		Number	*Milligrams*	*Percent*	*Percent*
Larvae	2 days	50	0.47	5.95	100
	1 month	50	.77		98
	2 months	50	1.53		86
	do	25	8.37	2.62	32
	3 months	25	4.85		60
	do	25	11.32		48
	do	25	13.47	2.70	32
	do	25	20.32	2.12	16
	4 months	25	5.46	2.71	48
	do	25	36.00		4

24-HOUR EXPOSURES

Larvae	Full grown	50	152.70	0.28	20
Prepupae		50		2.26	94
Pupae	0 to 2 days	50		.62	38
	6 to 8 days	50			40
	8 to 10 days	30		1.19	86.6
Adults	0 to 7 days	30		2.06	100

Full-grown larvae, prepupae, pupae, and adults were exposed to naphthalene vapor for a period of 24 hours (table 4). Forty-eight hours after exposure the larvae and adults were examined and the number of dead recorded. Those unable to crawl were considered as dead. The pupae and prepupae which survived treatments were allowed to develop into adults; those that developed into abnormal adults (undeveloped elytra and pupal abdomens) were recorded as dead. Full-grown larvae were the most resistant to lethal effects of naphthalene vapor and adults were the least resistant. The prepupae showed marked susceptibility to the vapor, whereas the pupae showed marked resistance until a few days before emerging as adults, when their susceptibility increased greatly. It is interesting to note that the stages which lost the greatest weight in 24 hours were the ones to which naphthalene was the most toxic. About 48 hours after treatment the affected pupae began to turn dark until they became chocolate colored, failed to respond when touched, and finally died. The reaction to naphthalene vapor was very gradual and the nervous and muscular systems were not the first tissues to be affected.

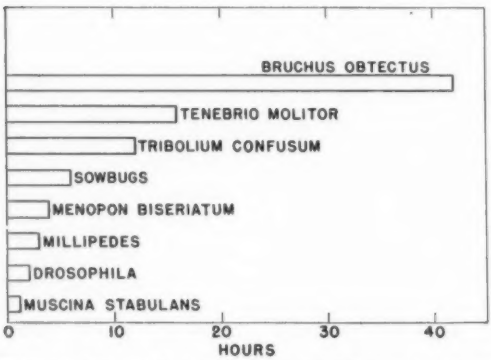

FIGURE 1.—The period of exposure required to kill approximately 100 percent of the species indicated.

Adults emerging from pupae subjected to sublethal dosages produced eggs which were not significantly different in number or fertility from the checks. A 6-hour treatment of adult mealworms had no effect on the fertility of eggs which surviving individuals laid, but it had a decided effect on the number produced. Control specimens produced 5.4 eggs per day per female, while in the treated lot 1.8 eggs per day per female were obtained.

For purposes of comparison several species of adult insects and closely related organisms were exposed to naphthalene vapors for varying periods of time and examined 48 hours after treatment. The time required to kill approximately 100 percent of the insects is shown in figure 1. Diptera were the most susceptible to naphthalene and Coleoptera the least susceptible of the insects used; in general it appeared that the most active insects were the most susceptible.

EFFECTS OF NAPHTHALENE INJECTIONS ON YELLOW MEALWORM PUPAE

It was thought that the injection of naphthalene into insects might yield some added information concerning its toxicity. Mealworm pupae were selected for this experiment because they were uniform in size (weight) and were easily handled.

Olive oil was used as a solvent for naphthalene since injection tests with olive oil alone had shown it to be nontoxic to the pupae when injected in much larger amounts than those used with naphthalene. The injection apparatus was similar to that used by Campbell (*1*). For making injections a rubber tube 8 cm long was attached to the pipette and a screw clamp was attached about 2 cm from the other end. The pipette was filled by suction and the screw clamp tightened. The pupae were held ventral aspect upward and the point of the glass needle was inserted near the lateral margin between the fourth and fifth abdominal segments. The point was directed cephalad for a distance of about 3 mm parallel to the longitudinal axes of the pupae. Pressure was then applied to the rubber tube by the thumb and forefinger which drove the solution into the pupae. The bore of the needle was too small for any blood to enter and the capillary attraction of the small bore prevented the solution from being sucked back when the pressure was released on the rubber tube. After every one or two injections the screw clamp was loosened to release the tension on the liquid in the capillary and then tightened again. The injected pupae were then placed in the constant-temperature cabinet for observation.

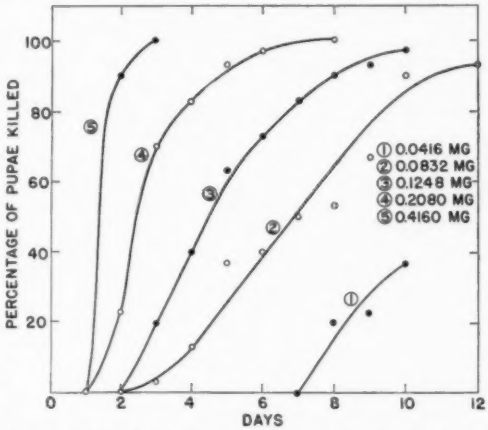

FIGURE 2.—The relation between the concentration of naphthalene injected into mealworm pupae, the rate of kill, and the total percentage of pupae killed.

Most of the injections were made without visible loss of blood. Pupae up to 2 days of age were used in all the injection experiments. Those that developed into abnormal adults were considered dead. Thirty pupae were used in each experiment. In the check, injected with olive oil alone, 10 percent failed to develop into normal adults. Figure 2 shows the relation between the concentration of naphthalene injected, the death rate, and the percentage of pupae ultimately killed.

The same toxic symptoms appeared in injected pupae as in pupae that were exposed for 24 hours to naphthalene vapor. Within a few days, the time depending upon the amount of material injected, the pupae began to turn dark but still responded to touch by wriggling. They lost all power of movement and were called dead when they finally became chocolate-colored. Examination of the body contents showed discolored plasma, fat, and muscles. All the tissues appeared to be undergoing disintegration. Naphthalene injected into the pupae did not produce materially different effects from those obtained with vapors.

From these injection experiments it seems that naphthalene vapor to be effective must be absorbed by the body tissues; that it is a

slow-acting poison to the pupae of the mealworm; and that it affects other tissues before it affects the nervous or muscular systems. The fat bodies were probably the first tissues to be affected since they appeared to be partly disintegrated and discolored in treated larvae and pupae.

SUMMARY AND CONCLUSIONS

A study of the toxicity of naphthalene to the bean weevil (*Bruchus obtectus* Say) and the yellow mealworm (*Tenebrio molitor* L.) in their various stages of development is reported.

Experiments with the eggs of the bean weevil and the mealworm showed that toxicity varied with the age of the eggs. It was found that the rate of respiratory metabolism of the eggs measured by the percentage daily loss in weight was directly related to lethal dosages of naphthalene. An increased loss in weight was associated with a greater toxicity of naphthalene to the eggs. The development of bean weevil eggs was noticeably retarded by sublethal exposure to naphthalene vapor. The developmental rate of mealworm eggs was not significantly affected by sublethal exposures to naphthalene. Eggs and very young larvae of the mealworm when fumigated with naphthalene turned reddish brown, the color intensity varying directly with the period of exposure.

Experiments with larvae of the mealworm showed that the toxicity of naphthalene decreased with increased age and weight of the larvae. Sublethal exposures of mealworm eggs or young larvae to naphthalene did not affect subsequent growth.

Resistance to naphthalene varied greatly between instars of the more advanced stages of the mealworm. The order of susceptibility of all the stages of the mealworm to naphthalene from least to greatest resistance was: (1) Eggs, (2) young larvae, (3) adults, (4) prepupae, (5) pupae, (6) mature larvae. Increased respiratory metabolism as measured by percentage daily loss in weight appeared to be related to increased toxicity of naphthalene with the different stages of the mealworm.

In experiments with several species of adult insects and closely related organisms Diptera were most susceptible to naphthalene while Coleoptera were most resistant.

Injections of olive-oil solutions of naphthalene into mealworm pupae produced the same toxic effects as when the pupae were exposed to naphthalene vapor. Naphthalene was a slow-acting poison to the pupae even when injected into the body. The nervous and muscular systems were not the first tissues to be affected, as shown by the wriggling movements of darkened, dying pupae when touched. Fat bodies were probably the first tissues to be affected since they appeared to be partly disintegrated and discolored in treated larvae and pupae.

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SOME ENVIRONMENTAL FACTORS INFLUENCING THE DEVELOPMENT OF HAIRY ROOT ON APPLE[1]

By A. J. RIKER, *professor of plant pathology*, D. H. PALMITER, *research assistant*, and E. M. HILDEBRAND, formerly *research assistant, Wisconsin Agricultural Experiment Station*

INTRODUCTION

The influence of certain environmental factors on the development of hairy root has been studied under partially controlled conditions. The need for such studies was indicated in a report by Riker and Hildebrand [2] on the seasonal development of hairy root on nursery apple trees grown from piece-root grafts. Various factors for infection and the development of disease appeared to be less favorable either at the beginning or at the end of the season than at midseason. Consequently, it appeared desirable to examine the relative importance of various soil temperatures and moistures when other factors were similar. Correlated with this work the effect of temperature on the growth of the causal organism, *Phytomonas rhizogenes* R. B. W. K. and S., was determined.

The objects of the present study were to measure the influence (1) of various soil temperatures on the development of hairy root, (2) of various soil moistures on the development of hairy root, and (3) of temperature on the causal organism.

MATERIALS AND METHODS

The host plants in these studies were 1-year-old root-grafted Fameuse apple trees, grown in southern nurseries and selected for uniformity. The trees varied in caliper from five-sixteenths to three-eighths of an inch and in height from 3 to 4 feet from the graft union. Before they were planted the roots were trimmed until they would fit into the containers used, and the tops were cut off at approximately 2 feet from the crown. As the trees grew, no more than two shoots were permitted to develop.

The experiments were all conducted in the greenhouse during the late winter and early spring months. This was the only time of the year when dormant trees could be planted and handled satisfactorily over a sufficient period of time at the desired soil temperatures.

The containers in which the trees were grown were cylindrical galvanized-iron cans 8 inches in diameter and 16 inches high. Two trees were planted in each can.

The soil moisture was regulated in the usual way by adding water until the lost weight was restored. The water was added both at the surface and through perforated aluminum tubes placed vertically in the middle of the cans. The sandy loam employed had a moisture-holding capacity approximating 40 percent of its dry weight. The

[1] Received for publication Dec. 16, 1935; issued June 1936. This work was correlated with a project carried on cooperatively between the Bureau of Plant Industry, U. S. Department of Agriculture, and the University of Wisconsin. It was supported in part by a grant from the special research fund of the University of Wisconsin. See legend fig. 1.

[2] RIKER, A. J., and HILDEBRAND, E. M. SEASONAL DEVELOPMENT OF HAIRY ROOT, CROWN GALL, AND WOUND OVERGROWTH ON APPLE TREES IN THE NURSERY. Jour. Agr. Research 48: 887-912, illus. 1934.

determinations were all made in percentages of the moisture-holding capacity of the soil. The various trials on soil moisture were made in a greenhouse where the temperature averaged about 24° C.

The soil temperature was regulated by placing the cans in Wisconsin soil-temperature tanks and adjusting the temperature of the water. The temperatures in the soil employed were 12°, 16°, 20°, 24°, 28°, and 32° C. The surface of the soil in every case was covered with an inch of ground cork to lessen evaporation and to maintain more uniform temperatures in the upper layers of soil. The soil moisture in the temperature series was 80 percent of the moisture-holding capacity of the soil in 1931, but was 75 percent in the later three trials. The tanks employed and the general procedure, except as noted, were similar to those used earlier by Riker.[3]

The cultures employed in these studies were the progeny of individual cells. Their previous history has been described by Wright et al.[4] under the numbers C–10, C–11, and C–12. They were introduced through scalpel cuts into the scions of the young apple trees after they had broken dormancy. The five inoculations in each tree were made well below the surface of the soil.

Three trials involving soil-temperature and soil-moisture series were run, respectively, during 1931, 1932, and 1933. Since difficulty was experienced with the 32° C. tank in 1931, and the temperature 28° had previously been found suitable and 32° unsuitable for the formation of crown gall on tomato,[3] particular attention was paid to these two temperatures in trials during 1934.

The numbers employed in the individual temperature trials were uniform. For each soil temperature during every season a single trial included eight cans with two trees each and five inoculations per tree. Thus every trial was based upon 80 inoculations. In each of the soil moistures employed four cans were used in 1933 and six cans in 1931, 1932, and 1934.

DEVELOPMENT OF HAIRY ROOT AT VARIOUS SOIL TEMPERATURES AND MOISTURES

The results of these studies were recorded in both the temperature and moisture series on (1) the growth in height of the host plant, (2) the percentage of infection by the hairy root bacteria, and (3) the growth measured by weight of the hairy root at the point of inoculation.

The records of the different trials in 1931, 1932, and 1933 have been averaged to secure the data presented. In 1931 no infection was secured in the 32° tank, which correspondingly lowered the final averages for incidence of infection and for weight of hairy root. In 1934 three tanks containing eight cans with two trees each were run at approximately 28° C. A similar set was run at 32°. Since these results corroborated the data presented at these temperatures, the details are omitted.

The growth of the host plant varied over the temperature range employed (figs. 1 and 2, *A*). It was greatest at 20° C., relatively

[3] RIKER, A. J. STUDIES ON THE INFLUENCE OF SOME ENVIRONMENTAL FACTORS ON THE DEVELOPMENT OF CROWN GALL. Jour. Agr. Research 32: 83-96, illus. 1926.

[4] WRIGHT, W. H., HENDRICKSON, A. A., and RIKER, A. J. STUDIES ON THE PROGENY OF SINGLE-CELL ISOLATIONS FROM THE HAIRY-ROOT AND CROWN-GALL ORGANISMS. Jour. Agr. Research 41: 541-547, illus. 1930.

vigorous at 16°, 24°, and 28°, and relatively poor at 12° and 32°. The growth of the host plant in the different soil moistures employed in 1932, 1933, and 1934 appeared to be approximately the same at 75 and 90 percent of the moisture-holding capacity, but was distinctly lower at 60 percent (fig. 3). The growth during 1931 in a trial with 40 percent moisture was quite inferior. The number of successful inoculations and the weight of hairy roots were also less. Other trials in 1931 at 60- and 80-percent moisture gave results comparable to those at 60- and 75-percent moisture during 1932, 1933, and 1934.

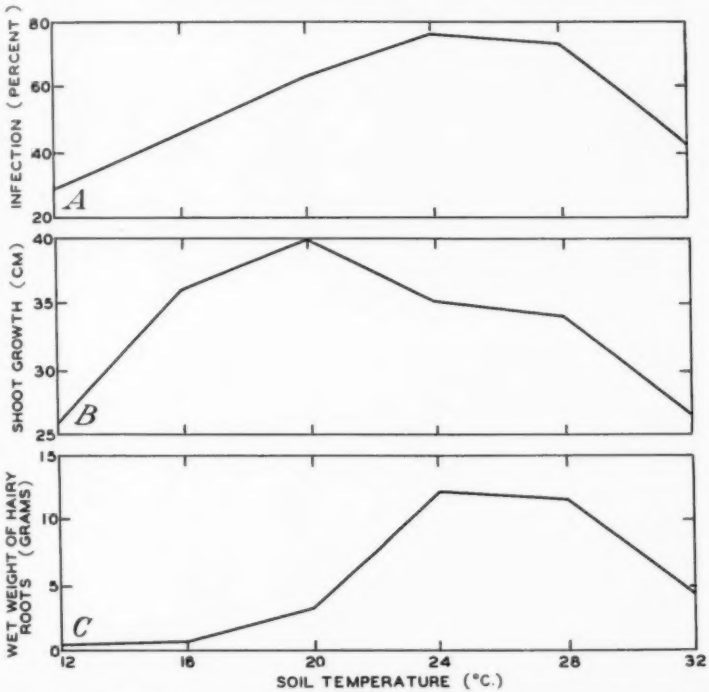

FIGURE 1.—The effect of soil temperature: *A*, On the percentage of infection on young apple trees by hairy root bacteria; *B*, on the length of shoot growth of the apple trees; *C*, on the amount of hairy root growth as measured by wet weight. All illustrations prepared by Eugene Herrling.

The percentage of infection by the hairy root bacteria was greatest at 24° and 28° C., and progressively less above and below these temperatures (fig. 1). The percentage of infection was slightly but not significantly greater at 75 percent of the moisture-holding capacity of the soil than at either 60 or 90 percent (fig. 3).

The growth of hairy root (fig. 2, *B*) following infection, as measured by wet weight approximately 10 to 11 weeks after inoculation, was greatest at 24° and 28° C., but progressively less at higher and lower temperatures (fig. 1). The growth of hairy root in the moisture series very closely paralleled that for growth of shoots. It was

relatively good from 60 to 90 percent; being approximately the same at 75 and 90 percent and slightly less at 60 percent (fig. 3). The development of hairy root, like that of crown gall [5], seems to be associated with the vigor of the host growth.

FIGURE 2.—*A*, shoot growth of young apple trees at soil temperatures of 12°, 16°, 20°, 24°, 28°, and 32° C. from left to right, respectively; *B*, hairy root growth at the same temperatures.

GROWTH OF HAIRY ROOT BACTERIA AT DIFFERENT TEMPERATURES

The growth of the hairy root bacteria was studied in both solid and liquid media at temperatures ranging, at 4° intervals, from 4° to 36° C., inclusive. Needle-point transfers were made to plates poured with carrot-extract agar so that seven equidistant colonies were grown in each Petri dish. Five dishes were grown at each temperature. The average diameters of these colonies after 16 days were taken as

[5] RIKER, A. J. See footnote 3.

the measure of growth at the different temperatures. The trials were made three separate times and all results averaged. Thus each point on the graph (fig. 4) represents the average diameter of 105 colonies.

Similar trials were made with yeast-infusion mannite agar that had the following composition: Magnesium sulphate ($MgSO_4.7H_2O$), 0.2 g; sodium chloride (NaCl), 0.2 g; calcium chloride ($CaCl_2$), 0.1 g; dibasic potassium phosphate (K_2HPO_4), 0.2 g; 10 percent yeast infusion, 100 cc; mannite, 10 g; agar, 17 g; and water to make 1 liter.

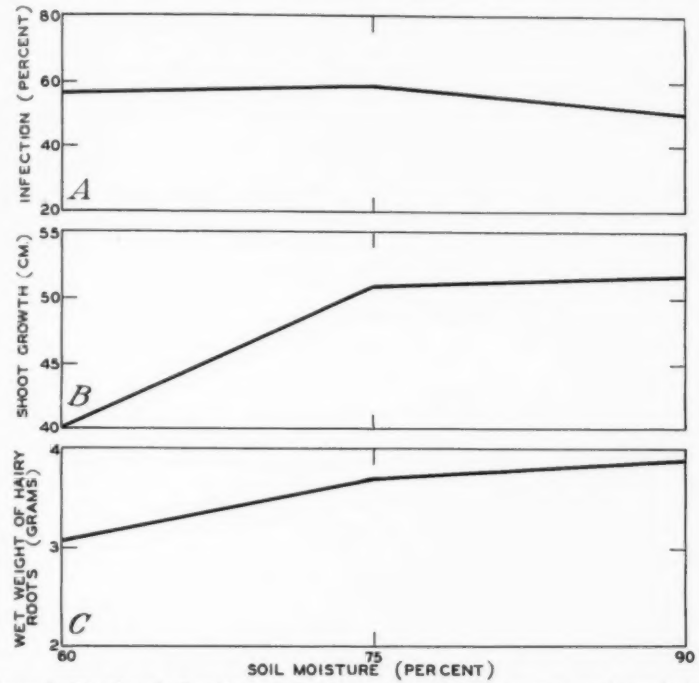

FIGURE 3.—The effect of soil moisture: *A*, On the percentage of infection on young apple trees by hairy root bacteria; *B*, on the length of shoot growth of the apple trees; *C*, on the amount of the hairy root growth as measured by wet weight.

The reaction was adjusted to pH 7.0. The results are shown in figure 4.

Similar trials were made with the yeast-infusion mannite media described above except that no agar was added. The amount of growth was measured by turbidity in relation to the McFarland [6] nephelometer scale. The results appear in figure 4.

These three different trials show that the largest amount of vegetative growth occurred at approximately 28°, although the growth was relatively good between 20° and 32° C. A slight amount of growth was found at both 4° and 36°.

[6] MCFARLAND, J. THE NEPHELOMETER: AN INSTRUMENT FOR ESTIMATING THE NUMBER OF BACTERIA IN SUSPENSIONS USED FOR CALCULATING THE OPSONIC INDEX AND FOR VACCINES. Jour. Amer. Med. Assoc. 49: 1176–1178, illus. 1907.

DISCUSSION

These studies assist in the interpretation of several results previously reported by Riker and Hildebrand [7], particularly in relation to the incidence of infection, incubation period, and rate of growth of apple trees and hairy roots.

The percentage of infection was greatest at 24° and almost as great at 28° C. This is at the upper limit for vigorous growth of the trees and is approximately at the optimum for the growth of the bacteria.

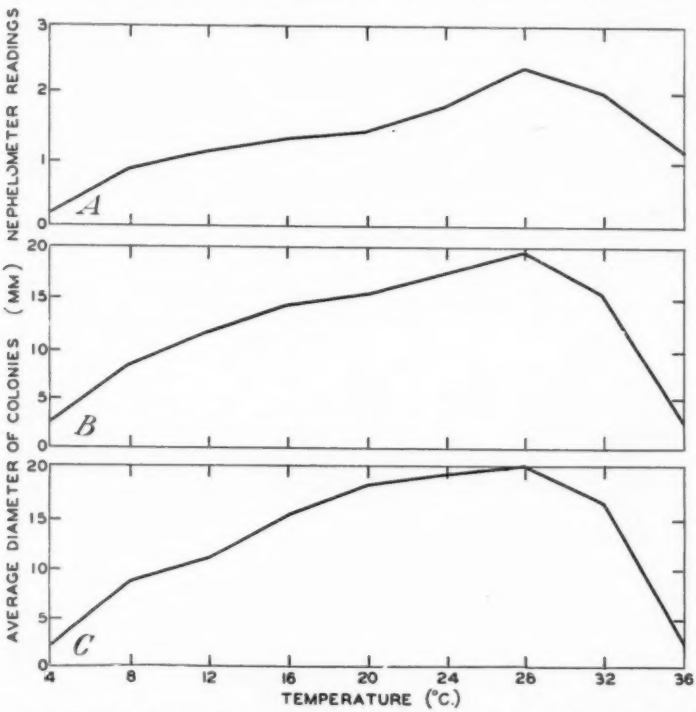

FIGURE 4.—The effect of temperature on the development of hairy root bacteria, as measured (*A*) by turbidity in tubes of liquid yeast-infusion mannite media and by the average colony diameters on (*B*) yeast-infusion mannite and on (*C*) carrot-extract agars.

The smaller percentage of infection at lower temperatures correlates with the low incidence of infection in the spring noted by Riker and Hildebrand when the apple grafts were inoculated at the time of grafting. However, these writers found that by midsummer the incidence of infection was very high. It seems likely from the symptoms of the hairy root observed at the lower temperatures that a much higher incidence of infection would have occurred if the inoculated trees either had been given a longer time or had been exposed to a higher temperature. Perhaps a similar explanation would account for the variation in the percentage of infection with varying soil

[7] RIKER, A. J., and HILDEBRAND, E. M. See footnote 2.

moisture; i. e., the incubation period was not long enough under the conditions to give the maximum percentage of infection.

The incubation periods defined and observed by Riker and Hildebrand were shortest during midsummer when the temperature was high and the trees were growing rapidly. Thus the environmental conditions that favor short incubation periods appear also to favor the growth of hairy roots.

The active development of hairy root was greatest at 24° and 28° C., temperatures at which the trees were growing vigorously and the bacteria were making maximum vegetative growth. Thus it appears that the short incubation periods are correlated with conditions favorable for active growth by both host and bacteria.

The growth of the apple shoots was relatively vigorous between 16° and 28° C., inclusive. Although the growth of both the host and the bacteria was relatively vigorous at 16° and 20°, the hairy roots grew more actively at the higher temperatures. No reason for this is apparent. The question might be raised whether the factors for root stimulation are present in larger quantity in this 24° to 28° range. In the moisture series the growth of the hairy root appears to be correlated with that of the host.

SUMMARY

Inoculations with *Phytomonas rhizogenes* were made on 1-year-old Fameuse apple trees kept at graduated soil temperatures and soil moistures. The highest incidence of infection, in the time of the experiment, appeared at 24° to 28° C., and at 60 and 75 percent of the moisture-holding capacity of the soil. This probably indicated the shortest incubation period. The growth of the shoots in length was greatest at 20°, with vigorous growth between 16° and 28°. Their growth was also more vigorous at 75 and 90 percent soil moisture than at 60 percent. The development in weight of hairy root was greatest at 24° to 28°. It was good over the entire range and only slightly greater at 75 and 90 percent moisture than at 60 percent. The air temperature was approximately 24°.

The growth of the hairy root bacteria was measured by colony diameter on yeast-infusion and carrot agar, and by turbidity in a yeast-infusion liquid medium, at temperatures from 4° to 36° C. Vegetative growth was greatest at 28°, good at 16°, 20°, 24°, and 32°, moderate at 8° and 12°, and slight at 4° and 36°.

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